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Extreme-Longevity Mutations Orchestrate Silencing of Multiple Signaling Pathways

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Abstract

Long-lived mutants provide unique insights into the genetic factors that limit lifespan in wild-type animals. Most mutants and RNA-interference targets found to extend life, typically by 1.5- to 2.5-fold, were discovered in *C. elegans*. Several longevity-assurance pathways are conserved across widely divergent taxa, indicating that mechanisms of lifespan regulation evolved several hundred million years ago. Strong mutations to the *C. elegans* gene encoding AGE-1/PI3K$_{CS}$ achieve unprecedented longevity by orchestrating the modulation (predominantly silencing) of multiple signaling pathways. This is evident in a profound attenuation of total kinase activity, leading to reduced phosphoprotein content. Mutations to the gene encoding the catalytic subunit of PI3K (phosphatidylinositol 3-kinase) have the potential to modulate all enzymes that depend on its product, PIP_3, for membrane tethering or activation by other kinases. Remarkably, strong mutants inactivating PI3K also silence multiple signaling pathways at the transcript level, partially but not entirely mediated by the DAF-16/FOXO transcription factor. Mammals have a relatively large proportion of somatic cells, and survival depends on their replication, whereas somatic cell divisions in nematodes are limited to development and reproductive tissues. Thus, translation of longevity gains from nematodes to mammals requires disentangling the downstream consequences of signaling mutations, to avoid their deleterious consequences.

Keywords

Lifespan; Longevity; *Caenorhabditis elegans*; Insulin; IGF-1 (insulin-like growth factor 1)

Introduction

Genetic mutations capable of extending metazoan lifespan were first discovered in the nematode *C. elegans* [1-8]. Mutations with substantial benefits to lifespan were later discovered in Drosophila, mice and rats, either by targeted quests for mutations in the corresponding pathway of these species [9-12] or by independent discovery [13-17]. Conserved mutations have also been found to increase “lifespan” of yeast, by several operational definitions [18-21].

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Gene mutations, if random, are expected to impair function far more often than improve it [22]. Thus, finding that mutations frequently augment lifespan (*e.g.*, see www.wormbase.org) implies that the mutated genes have *primary functions other than ensuring survival*. Genes that instead *reduce* longevity when they are mutated or knocked down have been termed "longevity assurance genes" (see [23,24]), on the assumption that their normal function serves to extend life. In this case, a possible trivial explanation is that they may affect essential functions or pathways, so that their impairment reduces physiological fitness. The appropriate test of a gene's role in longevity attainment is to assess whether life is extended through its over-expression. By studying single-gene mutations, RNA interference, and (more rarely) transgene overexpression, several signal-transduction pathways have been shown to modulate longevity [25-29]. Accumulating evidence indicates that these pathways intersect one another, to form a network of protective and regulatory genes that influence survival in diverse circumstances [28,30-39]. Environmental conditions, such as nutrients, stresses and pathogens, also impact lifespan. Although these "external" influences could perturb survival directly or by altering tissue metabolites, in several instances they have been shown to exert at least part of their effect through the same signal-transduction pathways that were demonstrated genetically to influence longevity [11,20,28,31,33,35,40-43].

A single-gene mutation in the nematode *C. elegans* was recently shown to increase adult survival by tenfold, exceeding the previous record by a factor of at least three [44]. Median lifespan was boosted from ~16 days at 20°C, to over 5 months, with comparable effects on mean and maximal (90^{th} percentile) lifespan. Four- to six-fold extensions of nematode lifespan had been achieved previously through a combination of two or three interventions [45-47]. Similarly, a ten-fold increase in yeast lifespan was recently attained by combining three interventions: two mutations plus severe caloric restriction [18]. These results suggest that in both taxa, several parallel mechanisms curtail normal lifespan, and that the benefits of subverting them are "additive" — meaning that their combined effect is roughly the sum of those seen for individual factors.

Nematode lifespan is limited by the normal operation of multiple genes

Reduction-of-function mutations that were discovered through mutagenesis screens, chiefly testing traits other than longevity, can individually enhance *C. elegans* lifespan by factors of 1.1- to nearly 3-fold. The first longevity-conferring mutation, an allele of *age-1*, came out of a sib-screen for long-lived mutants arising after chemical mutagenesis [1]. The temperature-sensitive (*ts*) *age-1(hx546)* mutant, when homozygous, extends life by 40-65% [8]. A very similar protocol, of EMS mutagenesis and sib screening for longevity, yielded only one gene, termed *age-2* [48]; a third mutagenesis screen, in which four stress-response assays served as surrogates for lifespan, produced only additional alleles of *age-1* [49]. These rather limited returns on sizeable investments of effort are all the more remarkable in view of the many longevity-enhancing mutations discovered by other means. The *daf-2* mutation, initially discovered as a *ts* mutant featuring constitutive production of dauer larvae at ≥25°C [50], was much later tested for adult lifespan and found to extend its normal duration by almost two-fold [6]. When 15 independent *daf-2* alleles were crossed into an isogenic background, they extended normal lifespan to quite variable degrees: from as little as 10%, up to 2.5-fold [51]. The protein encoded by *daf-2* is a membrane receptor-kinase responsive to many insulinlike ligands (both agonists and antagonists), whereas the *age-1* product is the p110 catalytic subunit of class-I PI3 kinase ($PI3K_{CS}$), responsible for converting PIP_2 to PIP_3. These two kinases participate in the insulin/IGF-1 signaling (IIS) pathway of nematodes, which regulates dauer formation, fertility, stress response, and lifespan. Further downstream, and excluded from the nucleus by phosphorylation via the DAF-2/AGE-1/PDK-1/AKT kinase cascade, lies DAF-16/FOXO — a Forkhead or winged-helix transcription factor encoded by the *daf-16* gene. When IIS is blocked, DAF-16 enters the nucleus to regulate transcription of several hundred genes,

many of which are involved in survival and stress responses. All known phenotypes of either *age-1* or *daf-2* mutations can be largely or entirely reversed by a second mutation inactivating *daf-16* [30,44,45,52,53]. Rescue experiments, restoring wild-type *daf-2* or *daf-16* to individual cell types in mutant worms, demonstrate that IIS effects on lifespan are tissue specific [54, 55], and imply critical roles of neurons and gut in the regulation of nematode longevity.

Of all the life-prolonging mutations discovered in the nematode *C. elegans*, until recently none had surpassed the 1.5- to 3-fold increases reported over a decade ago for disruptions of the insulin/IGF-1 signaling (IIS) pathway [5,45,51,52]. Checking for convergence with the IIS pathway has thus become a "default test" for all new longevity mutations, to determine whether they are dependent or independent of DAF-16. Once IIS and other pathways were shown to extend lifespan in the nematode when disrupted, they were cross-checked in short order for corresponding effects in other species. In general, similar mutations have produced more modest life extensions in other taxa, and in many cases no effects were seen. In Drosophila, very few single-gene mutations increase longevity by more than 2-fold [10,56], and none have exceeded 1.5-fold in mice [9,10,12,13,16,57,58].

Greater extensions of lifespan can be attained by combining several interventions. In particular, specific pairings of mutated alleles for *daf-2* and *daf-12* (encoding a nuclear hormone receptor) can increase nematode longevity by 3.5-fold [45] or 4.4-fold [51]. Multiple interventions, such as a *daf-2* mutation combined with germ-cell ablation or caloric restriction, can increase *C. elegans* lifespan by 4- to 6-fold [46,47]. In mice, a mutation impairing pituitary development extends lifespan by 35–40%; when combined with caloric restriction, it yielded a record-setting 1.8-fold extension [16]. Thus, there exist in each species multiple pathways for life extension, effects of which are largely additive. This is often assumed to imply their independence, although "additivity" can also arise in a single pathway if each of the constituent mutations disrupts it only partially.

Several clear patterns have emerged. As longer-lived species are examined, the gains to be reaped from interventions are diminished. This is precisely what would be expected if longevity is subject to natural selection like other life-history traits: longer-lived species presumably have already been subjected to selective pressure to establish life-extending mutations as the norm. Another recurring observation is that long-lived mutations generally confer resistance to stresses [15,59,60]. However, several natural genetic variants for lifespan, quantitative trait alleles, vary widely in resistance to specific stresses [61], so it may be unwise to rely on any single stress-resistance test as a short-term surrogate for assessing longevity [49,60].

Null mutations in the *age-1* gene extend *C. elegans* lifespan by ten-fold

C. elegans strains carrying a nonsense mutation of the *age-1* gene (either the *mg44* or the *m333* allele) cannot be maintained as homozygous-mutant lines, but instead are propagated as heterozygotes, in which the mutated gene copy is offset by a wild-type allele on a "balancer" chromosome, which also carries visible mutations as markers. These worms self-fertilize (*C. elegans* reproduction being primarily hermaphroditic), to yield roughly one-quarter of total progeny homozygous for the *age-1* mutation. These *age-1* homozygotes may be loosely termed the "F1" generation, and their progeny would then be "F2" homozygotes. F2 mutants for these nonsense alleles, although genetically identical to their F1 parents, are clearly quite different in many respects. They develop very slowly at 15° or 20°C, and their development arrests completely at 25°C [44]. The adults are far more resistant to both electrophilic and oxidative stresses, and live 10-fold longer than normal, wild-type worms under benign conditions ([44] and Figure 1). Their lifespans are uniformly extended, with 25th, 50th (median), 75th and 95th percentiles all nearly 10 times those seen in near-isogenic worms bearing wild-type *age-1* alleles.

In appearance and activity, they resemble normal worms at a tenth their age. The F1 homozygous generation, their parents, show intermediate stress resistance [44] and roughly a doubling of wild-type lifespan [62]. The dramatic difference between these two generations, despite identical genes, is almost certainly a result of normal components in the egg cytoplasm that gives rise to F1-homozygous worms. The parent that produced this cytoplasm was an *age-1/+* heterozygote, which would have produced essentially normal *age-1* mRNA, PI3K enzyme, and its PIP_3 product.

The *age-1* gene of *C. elegans* encodes the catalytic (p110) subunit of phosphatidylinositol 3-kinase. The PI3K enzyme family transfers a phosphate from ATP to the 3 position of the inositol ring at the head of phosphatidylinositides. Specific PI3K enzymes convert phosphatidylinositol (abbreviated PtdIns or PI) to the monophosphate, PtdIns(3)P; PtdIns(4)P to PtdIns(3,4)P_2; and PtdIns(4,5)P_2 (PIP_2) to PtdIns(3,4,5)P_3 (PIP_3) (Figure 2). The mammalian tumor-suppressor gene *Pten* encodes the Pten phosphatase which removes the 3-phosphate from PIP_3., thus opposing PI3K action. PTEN in nematodes, like Pten of mammals, converts PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2, and PtdIns(3,4)P_2 to PtdIns(4)P [63-65]. Mammals also have SHIP1 and SHIP2 phosphatidylinositol 5-phosphatases, which can deplete PIP_3 by removal of the 5-phosphate to form PtdIns(3,4)P_2. There are three classes of mammalian PI3K (I, II, and III), which differ in substrates, structure and activators. The *C. elegans* AGE-1 protein is a class-I PI3K catalytic subunit, based on its sequence and structure, which serves to convert PI(4,5)P_2 to PI(3,4,5)P_3.

All class-I PI3K enzymes are heterodimeric, consisting of a catalytic (p110) subunit, of which mammals have four varieties [65] but *C. elegans* has only one (AGE-1; see WormBase); and a regulatory subunit, of which mammals have seven known varieties [65], while worms are only known to have one, AAP-1 (WormBase). AGE-1 most closely resembles p110-α and -β catalytic subunits [62], narrowing its classification to class IA. Because nematodes have fewer PI3K varieties than mammals, AGE-1 may share some properties of mammalian class-IB PI3Ks. F39B1.1 encodes another *C. elegans* p110 protein, which is more closely akin to a mammalian class-II PI3K. The third nematode PI3K p110 gene, *vps-34*, is an ortholog of the yeast *vps34* gene, encoding a class-III $PI3K_{CS}$. Class-III PI3Ks are implicated in vesicular trafficking, autophagy, endocytosis and secretion [66]. In keeping with this, yeast VPS34 governs endocytosis, and *C. elegans vps-34* is thought to be involved in endocytic trafficking between cell compartments, including that of low-density lipoprotein (LDL) receptors [67]. Class-II and -III PI3Ks phosphorylate the 3 position of PI(4,5)P_2 with very low efficiency *in vitro*, where they show a marked preference for other PIP substrates [62]. If some substrate promiscuity also occurred *in vivo*, it would blunt the phenotype of *age-1*-null mutants.

Roles of phosphatidylinositol 3,4,5-phosphate (PIP_3) in signal transduction

Under normal circumstances, PtdIns(3,4,5)P_3 (PIP_3) is roughly a thousand-fold less abundant than its precursor, PtdIns(4,5)P_2, which is itself a quite scarce molecule. PIP_3 levels increase up to 100-fold in activated cells [68], and are detectable in nematodes only after they are induced by starvation or oxidative stress [69]. The exceedingly low levels of this molecule, together with the fact that PTEN (which removes the 3-position phosphate of PIP_3) acts as a tumor suppressor in mammals, imply that PIP_3 is far from innocuous and must be stringently controlled to prevent undue cell proliferation. The role of PI3K in IIS is believed to proceed entirely through PIP_3 generation [64,65,70], although the p110 catalytic subunit also has protein-kinase activity, as demonstrated by phosphorylation of its regulatory subunit [34,71]. PIP_3 molecules carry two fatty-acid chains (R_1 and R_2 in Figure 2) that anchor it to the inner cell membrane. The hydrophilic phosphatidylinositol headgroup projects into the cytoplasm where it is avidly bound by proteins possessing a pleckstrin-homology (PH) domain. IIS proteins that dock to PIP_3 include PDK-1 (phosphoinositide-dependent kinase 1), AKTs, and

SGK-1 [65,72]. Once they are tethered to the membrane, the IIS components are effectively constrained to the same 2-dimensional surface, greatly promoting their interaction by elevating their effective local concentrations with respect to one another.

Tethered PDK-1 phosphorylates AKT-1 at a Thr residue, thereby activating it and allowing the AKT complex to phosphorylate the DAF-16/FOXO transcription factor. The latter is an inhibitory modification, restraining DAF-16 to the cytoplasm where it cannot act on its target genes [52,73]. PIP_3 not only tethers the AKT complex to the membrane, but its binding to the AKT-1 pleckstrin-homology domain also allosterically exposes a phosphorylation site to PDK-1, which is thus able to activate AKT-1 [65,72]. Thus, in addition to a stoichiometric requirement for PIP_3 to enable anchoring of AKT-1 (and many other signal-transduction molecules) to membranes, PIP_3 also may play an essentially catalytic role to transiently alter the conformation of AKT-1 (and possibly other targets), thus permitting their site-specific phosphorylation. We hypothesize that PIP_3 dissociates and is subsequently bound by other target molecules, in which case a single molecule of PIP_3 may be sufficient to activate all of a cell's AKT-1, although more PIP_3 would be needed for membrane-tethering of AKTs and other targets. Insofar as the anchoring role of PIP_3 is a quantitative one (working, in effect, by mass action), whereas the allosteric requirement for activation is absolute, it is likely that removing the last traces of PIP_3 from a cell would block the last vestiges of AKT-mediated signaling, and also any other pathways in which PIP_3 plays a comparable allosteric role.

This may account for the markedly enhanced survival of F2 homozygous *age-1(mg44)* mutants relative to their F1 parents, in which there still retain traces of PIP_3 carried over from their heterozygous parents. If so, then the secret of extreme longevity may be to remove any remaining traces of PIP_3-dependent signaling — a state which only allows *C. elegans* to develop, quite slowly, at lower temperatures. In higher eukaryotes, with requirements for cell division not only during development but also in specific adult tissues, this will be far more challenging to achieve without sacrificing fitness under some circumstances.

Loss of PI3K disrupts multiple protein-kinase activities

We assessed kinase activity in normal and *age-1*-mutant worms using an *in vitro* phosphorylation assay. As illustrated in Figure 3, F2 *age-1(mg44)* lysates show remarkably low kinase activities toward endogenous substrates — less than 8% of levels seen in wild-type worms. Activity was also low in the F1 generation, although higher than in F2 worms ($P<0.05$; Figure 3c). Steady-state levels of phosphoproteins were accordingly depressed in F2 adults, by >40% relative to controls [30]. Kinase activity was also reduced ~30% in *age-1(hx5456)*, a widely-studied but weaker mutant allele (Fig. 3c). The decline in *hx546* appears to be entirely mediated by DAF-16/FOXO or other transcription factors further downstream, since it is reversed in double mutants also lacking functional DAF-16/FOXO. However, only about half of the deficit is reversed in *age-1(mg44)* F2 adults, implying that this strong-mutant allele employs both DAF-16/FOXO-dependent and -independent routes (Figure 3, c and d). The F2 mutants are sterile, and consequently for comparison the other strains were all assessed when post-gravid. The presence of residual eggs in other strains could not account for the >12-fold decrease in kinase activity of F2 *age-1(mg44)* adults, since $N2_{DRM}$ eggs actually contain less activity per μg of protein than do $N2_{DRM}$ adults (Figure 3d).

Loss of PI3K leads to transcriptional inhibition of multiple signaling pathways

If kinase attenuation in *age-1* mutants were only due to the absence of PIP_3 needed to anchor or activate cellular kinases, it should be unaffected by loss of DAF-16/FOXO. As noted in the preceding section, this is clearly not the case. The deficit in *age-1(hx546)* kinase activity is fully reversed by a second mutation to the *daf-16* gene (in fact it is “over-reverted”; see Figure

3c), while the same mutation restores just half of the kinase deficiency seen in F2 *age-1 (mg44)* worms. To test for transcriptional regulation of IIS kinases, we used RT-PCR (real-time polymerase chain reaction) to quantify transcript levels of several IIS components. The data (Figure 4) provide compelling evidence for transcriptional silencing of IIS moieties in very long-lived F2 *age-1* adults, while the weaker *hx546* allele produced much less attenuation. Most of this inhibition, although not all, disappears in double mutants also defective for *daf-16* — again implying that transcripts are attenuated by multiple routes.

Profound silencing is seen, in F2 *age-1(mg44)* adults, for genes encoding the insulin receptor, DAF-2 (**a**); all 3 $PI3K_{CS}$ classes: AGE-1, F39B1.1, and VPS-34 (**b**, **c**, **f**); SGK-1 (**e**) and PDK-1 (**g**) kinases that phosphorylate AKT-1 (although AKT-1 itself (**h**) is not transcriptionally modulated), and the DAF-18/PTEN phosphatase (**d**) that opposes AGE-1 kinase. As a result of disrupted IIS inhibition of DAF-16/FOXO, two of its positive target genes, *sod-3* (**i**) and *pepck* (**j**), are strongly induced in *age-1(mg44)* F2 worms.

Intersections among pathways

Signal transduction pathways do not operate in isolation, but cross-talk with multiple other pathways [34,35,73-75]. Their interactions are complex, and are perhaps best viewed as a multidimensional fabric that can be “tugged” in many directions by various inputs [30]. Crosstalk is especially well documented between the IIS and the JNK and p38/MAPK stress- and cytokine-response pathways [35,74], invoking interactions with AMP-activated kinase (AMPK) and TOR complexes (see Figure 5). IIS is initiated by a membrane kinase-receptor, which responds to many insulinlike peptides, both agonists and antagonists [76]. In mammals, secretion of insulin is governed by Wnt/β-catenin signaling, and β-catenin coactivates FOXO [37]. The regulation of other insulin-like peptides, however, remains largely unexplored.

As an instructive example, AKTs can be activated by multiple inputs, including DNA-dependent protein kinase (DNA-PK) [77,78]. Multiple pathways converge through their common activation of AKTs, and AKT outputs are also diverse, utilizing many routes in addition to DAF-16/FOXO. In mammals (and presumably in other taxa), the critical PDK-1 phosphorylation of its target Thr in AKT-1 requires both a prior Ser phosphorylation, usually by TORC2 (target of rapamycin, complex 2), and also membrane- docking to PIP_3 by the AKT-1 pleckstrin-homology domain [65]. AKTs directly activate at least 14 proteins, including IRS-1 (providing feedback reinforcement of its own activation), RAF-1, eNOS, NFkB, and several cell-cycling and anti-apoptotic genes. Indirectly, they promote synthesis of proteins (via TSCs and TORCs) as well as glycogen (via GSK-3). In addition, AKTs inhibit at least 11 mammalian targets, including FOXO, androgen receptors, and pro-apoptotic gene products [34,79,80].

The strong *age-1(mg44)* mutant allele shows transcriptional downregulation of genes encoding DAF-16-interacting factors SMK-1, PAR-5 and SIR-2.1; of TGF-β type I and II receptors DAF-1 and DAF-4, as well as the SMAD transcription factor DAF-3; of AMPK/TOR pathway components AAK-1/AMPK (but not AAK-2), LET-363/FRAP, and DAF-15/RAPTOR; and of ERK/MAPK components LET-60/RAS, LIN-45/ERK, MEK-1/ERK, MPK-1/MAPK, glycogen synthase kinase GSK-3, and the SKN-1 transcription factor. These inhibitions of gene expression are all reversed, at least in part, in *daf-16; age-1* double mutants [30].

DAF-16/FOXO has been implicated as a *central convergence point* for diverse pathways, in several taxa separated by considerable evolutionary distances. Nuclear entry of DAF-16/FOXO is blocked by phosphorylation of at least 3 sites by the AKT complex and/or SGK-1. Stranded in the cytoplasm, this transcription factor is inactive and may be degraded. Nuclear exclusion is the principal, and perhaps sole, means of DAF-16/FOXO regulation through the IIS kinase relay. Several additional mechanisms are now known, by which *non-IIS* pathways can affect

DAF-16/FOXO, thereby converging with IIS to modulate longevity and stress responses. At least 6 sites on DAF-16 are phosphorylated by AMP-activated protein kinase (AMPK), activating a specific subset of transcriptional targets that include a number of signal-transduction and oxidative-stress-resistance genes [42,81]. Crosstalk between IIS and nutrient sensing thus takes place within DAF-16 (or FOXO of mammals). Proteins that bind to DAF-16 can alter its nuclear entry, as do SIR-2 and 14-3-3 proteins [82,83], or serve as transcriptional coregulators such as SMK-1 [84] and β-catenin [28]; whereas HSF-1 is an independent transcription factor that nevertheless interacts with DAF-16 [85,86]. PHA-4 provides a particularly instructive example of a factor that acts in parallel to DAF-16, and yet interacts with it indirectly through competition for SMK-1, a shared coactivator [87]. Protein : protein interactions may be sensitive to site-specific modifications of DAF-16, which include phosphorylation [82,83], acetylation [31,88], as well as ubiquitinylation, which alters DAF-16 activity through proteasomal degradation [89]. Although distinct from IIS, all of these interactions obviously depend on the presence of DAF-16; nonetheless, there could be parallel effects on other transcription factors, which persist in its absence [28,82,85,87]. Thus, partial reversion of *age-1(mg44)* F2 traits by a second mutation to *daf-16* (Figures 3 and 4) cannot be taken as evidence that AGE-1 acts only through the canonical IIS pathway.

The pervasiveness of crosstalk among pathways could be a consequence of the relatively limited repertoire of cellular responses to a much more varied range of stimuli. Cross-talk provides a concerted response to diverse signals arriving in an unpredictable sequence. The integration of signaling cascades could be compared to a neural-network algorithm for computer learning, in that the association between inputs and outputs cannot be defined by a set of rules.

The unprecedented improvement in longevity and stress resistance, seen in *age-1(mg44)* F2 adults bearing a single mutated gene, might be attributed to the profound realignment of signaling pathways when PIP_3 is absent. The mechanisms affected clearly extend beyond those known to be directly entrained by IIS. Although IIS in *C. elegans* has often been portrayed as a linear pathway (supplemented by crosstalk to Sir2, MAPK, TOR, and AMPK), the central role of PIP_3 depletion and the interplay with other pathways imply a distinctly nonlinear structure. Feedback loops, acting through both insulinlike peptides [90] and transcriptional inhibition of many kinases by DAF-16/FOXO [30], imply a more complex circuitry that cannot be adequately described as a collection of one-dimensional reaction chains. Most critically, the more elaborate the circuit, the greater the potential for the system to “hang” or become locked in a state of chronic repression and unresponsiveness to diverse signals (see Figure 6, adapted from [30]).

Tissue-specific determinants of metabolism and lifespan

D. melanogaster lifespan is increased when Cu/Zn-SOD transgenes are targeted to motor neurons, but not to other tissues or expressed globally [91]. This was interpreted to mean that lifespan is limited by oxidative damage to the nervous system, but that SOD overexpression in other tissues is not beneficial to survival, or may even oppose it. IIS modulation of *C. elegans* longevity also hinges on neurons. Mutations impairing *daf-2* (which encodes the insulin/IGF-1 receptor) or *age-1* (encoding PI3K) increase lifespan [6,8,92-95], whereas this effect is reversed when wild-type *age-1* or *daf-2* transgenes are targeted just to neurons (but not when they are expressed in muscle or the digestive tract) [54]. This implies that worm longevity is limited by metabolic activity or signaling of neurons. Effects of *age-1* and *daf-2* mutants on lipid metabolism, however, are most potently reversed when the corresponding wild-type genes are expressed in muscle cells [54]. Nerve and intestine cells are responsible for making most of the worm's INS-7, the one insulin-like peptide that has been demonstrated

to limit lifespan [90]. Longevity enhancement by mutations that disrupt sensory-neuron function [4] also indicates that neurons play a key role in setting *C. elegans* lifespan,

Mouse lifespan increases when *Igf-1R* signaling is suppressed globally [12], implying a similar role for Igf-1 signaling in mammals. Mice in which one copy of the *Irs2* gene has been knocked out, whether globally or only in the brain, live 17–18% longer than controls [96]. Although this establishes that IIS attenuation in the brain is sufficient to extend life, it is not known whether the signaling involves receptors for insulin or Igf-1, or hybrid receptors [97,98]. Irs-2 was long thought to respond exclusively to insulin receptor [96], but Igf-1R has been shown to phosphorylate both Irs-1 and Irs-2 [32,99]. This could be viewed either as Igf-1 signaling through Irs molecules [97,98], or as inter-pathway crosstalk, just as Jnk, Ampk, and S6k impinge on IIS through Irs phosphorylation [35,43,100]. PI3K p110γ can be deleted without extending life, possibly because this isoform is normally expressed only in macrophages and a few other tissues, where its absence interferes with innate immunity and inflammation [101].

If this were translated to mammals, could humans (like Methuselah) surpass 900 years?

Invertebrate models have led to the discovery of new genetic pathways, and also of unexpected consequences from altering known pathways and genes. Advantages of these model systems include the ease of conducting genetic studies, large-scale screens, and even selection experiments. Moreover, a relatively brief lifespan permits direct assessment of longevity where the equivalent study in any mammalian species would be far more costly and time consuming.

Invertebrate systems have been validated as useful models for human aging and age-associated debilities, by overwhelming evidence that at least some mechanisms determining lifespan are remarkably conserved from nematodes to mammals. Strong *age-1* mutations, which increase worm lifespan ten-fold, are central to insulin/IGF-1 signaling. IIS is well established as a highly and broadly conserved pathway which extends life when abrogated in nematodes, insects and mice. Mechanisms may thus also be conserved, underlying the effects of extreme IIS disruption in the worm, but it will be neither simple nor straightforward to translate them for the benefit of mammals. That would only be possible if an intervention could be applied selectively to just nondividing cells so as to avoid lethality and cell-cycle arrest in essential mammalian cell compartments.

A two-fold or ten-fold increase in nematode lifespan may not, however, translate to a proportional enhancement of human survival. Extrapolation from worms to mammals is risky at best, and it cannot be assumed that interventions will result in comparable life extension factors. Longevity gains from dietary restriction, or from mutations studied previously, yield smaller benefits to Drosophila than to nematodes, and smaller still to mammals. This is not unexpected, since mammals have evolved to live many times the worm's lifespan, and humans live nearly twice as long as the next longest-lived primate. From an evolutionary perspective, mammals and their ancestors have already undergone several hundred million years of natural selection favoring traits that could directly or indirectly favor increased longevity, and may thus have already settled on gene sequences that promote lifespan. Moreover, the very notion of a “life-extension factor” that could apply across taxa presumes a linear response rarely seen in biology.

Even if tenfold life extension, realized in nematodes and yeast, proves to be unattainable in humans, there is still ample room for pharmacological extension of healthy human life beyond present levels, and even beyond what could be achieved by dietary restriction or more modest attenuation of IGF-1 signaling. This possibility, first hinted in the nematode, would perhaps

never have been indicated by studies of mammals or any other metazoan dependent on continuing cell division, since AKT signaling is essential for cell replication. Substantial improvements in healthy lifespan may be achievable, but will require striking a delicate balance between two opposing cell states, replication and quiescence.

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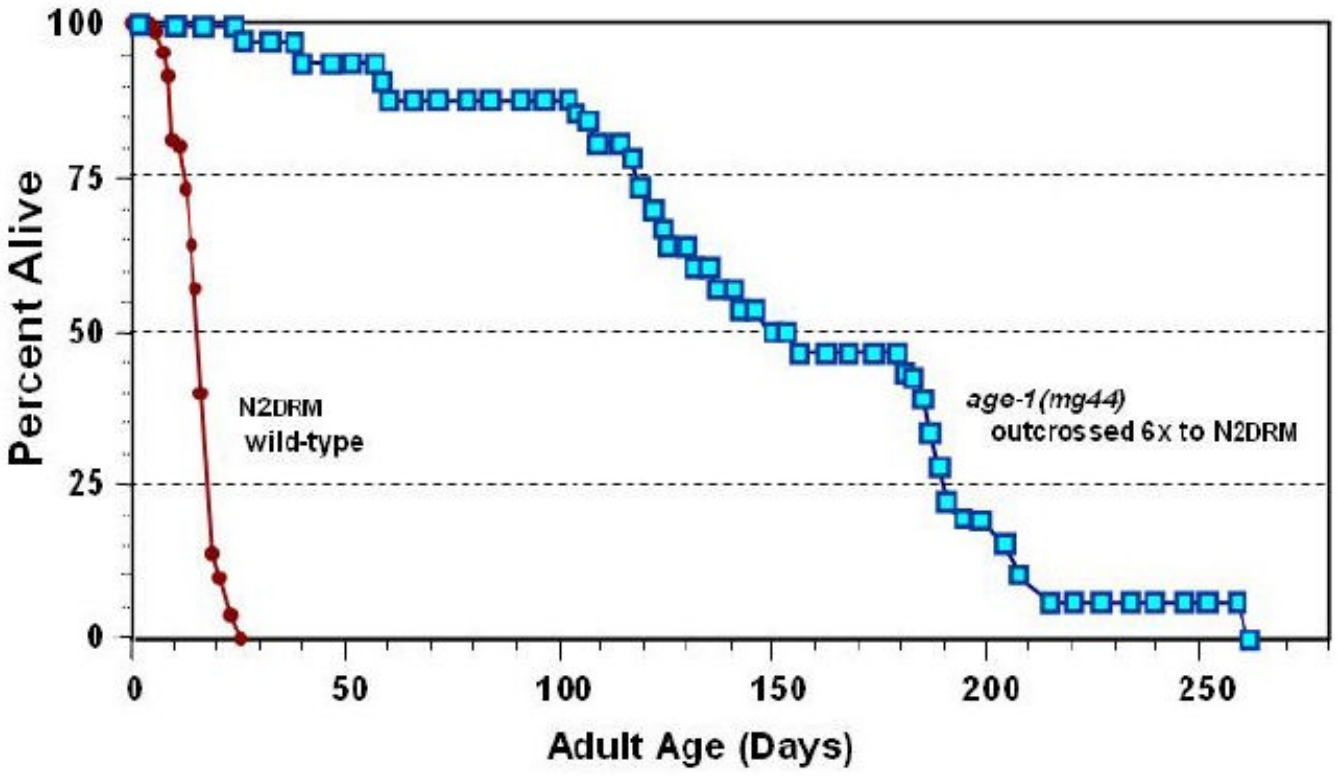

Figure 1. Survivals of F2 *age-1(mg44)* homozygotes *vs.* N2DRM controls

Survivals are plotted for *age-1(mg44)* F2 (squares) and N2DRM (circles). (Redrawn from [44]).

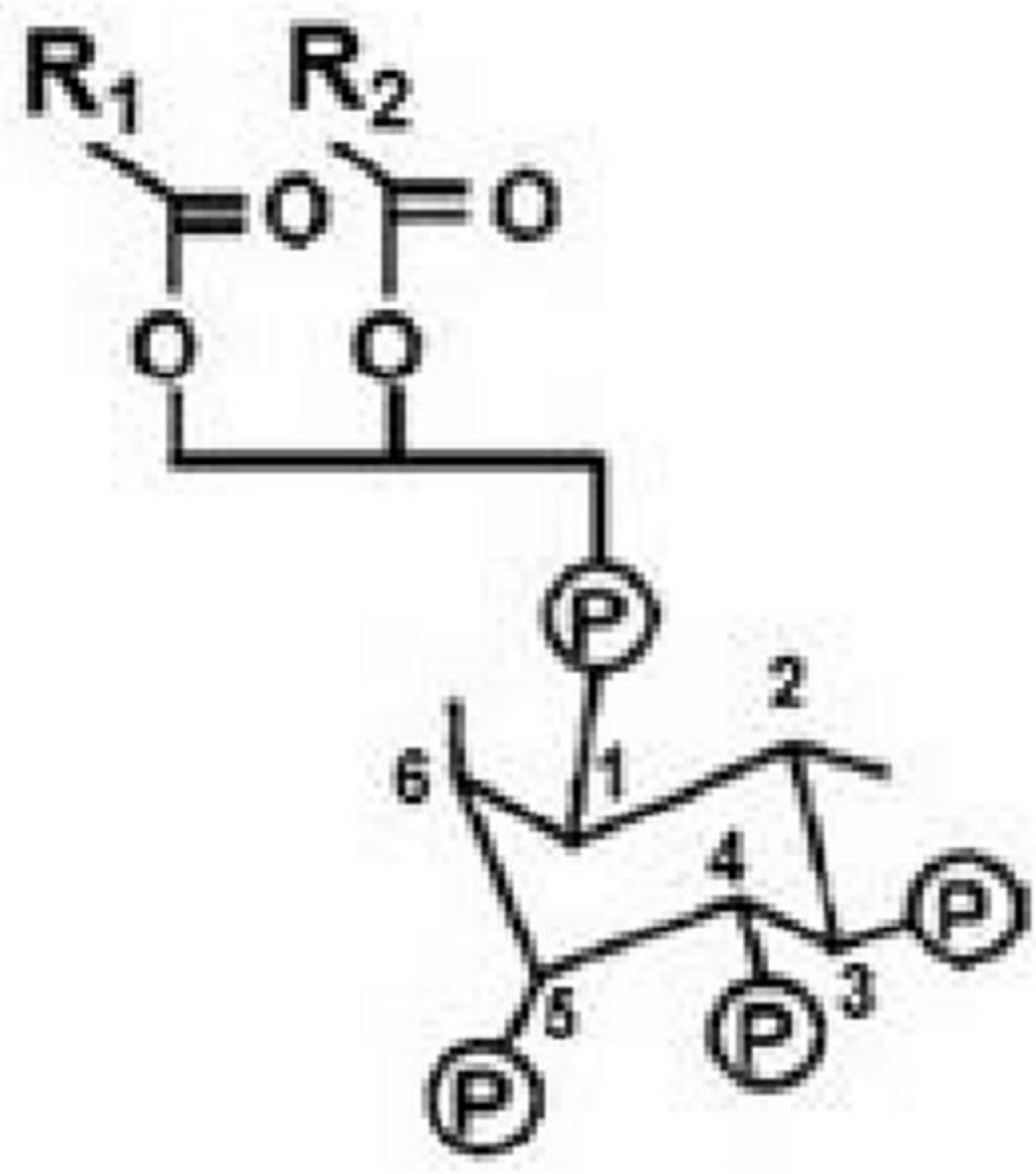

Figure 2. Structure of PIP_3

R_1 and R_2 are fatty-acid chains that vary among different molecules.

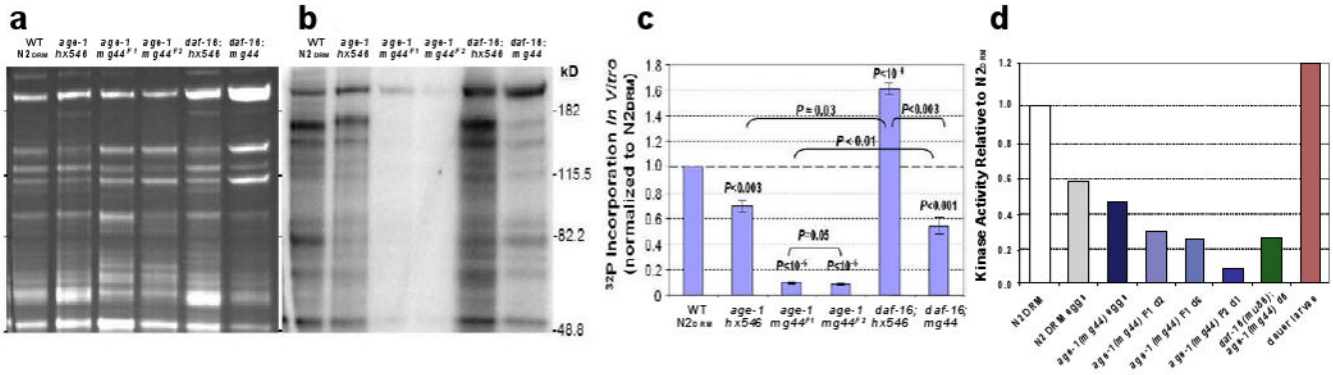

Figure 3. Protein-kinase activity for endogenous substrates are reduced in *age-1(mg44)* F2 homozygotes

Day-6 adults were harvested, frozen in liquid nitrogen and ground over dry ice. Kinase activity of cleared, sonicated lysates was assessed by γ-^{32}P-ATP incorporation per 20 μg protein sample, in 1 min at 30°C. Samples were electrophoresed on gels of 10% acrylamide/SDS. (**a**) Gel stained with SYPRO Ruby (invitrogen) for total protein. (**b**) ^{32}P β-image (Molecular Dynamics Storm) from the gel in **a**, dried under vacuum. (**c**) Data summary from 2–3 independent expansions each, of strains N2$_{DRM}$, *age-1(hx546)*, F1 *age-1(mg44)*, F2 *age-1 (mg44)* homozygotes, and *daf-16* double mutants with each *age-1* allele. (**d**) Additional controls show that *in vitro* kinase activity is lower in F2 homozygotes of *age-1(mg44)*, even as day-1 adults, than in F1's at adult days 1 or 6; a second mutation, deleting most of the *daf-16* gene, restores *age-1(mg44)* kinase activity to F1 levels; eggs laid by N2DRM or *age-1 (mg44)* F1 adults are intermediate in kinase activity; and dauer larvae have even higher kinase activity than N2DRM adults. Adapted from [30].

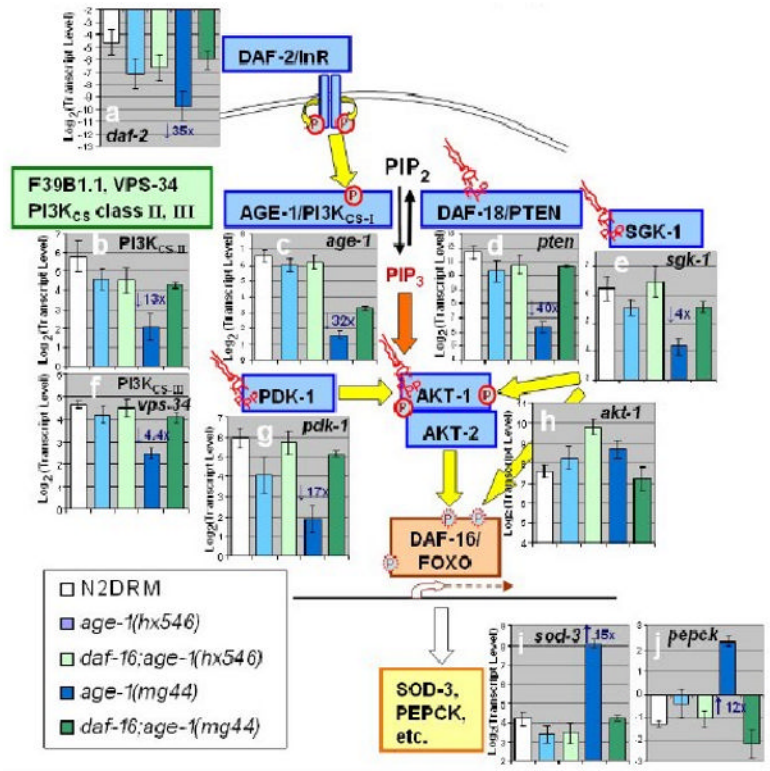

Figure 4. Transcriptional suppression of IIS genes in *age-1(mg44)*

Transcript levels were assayed by real-time polymerase chain reaction (RT-PCR). Expression histograms are shown superimposed on a schematic diagram of IIS. Yellow arrows show protein phosphorylations (circled P's); orange arrows indicate binding of phosphatidylinositol 3,4,5-triphosphate (PIP3, "structural" symbols). Within each histogram, transcript mean ± SEM (steady-state) is shown on a log(2) scale, comparing wild-type to 4 *age-1* mutant groups and to dauer larvae. For each group, fold changes are shown (*e.g.*, "3×"), of *age-1 (mg44)*-F2 relative to $N2_{DRM}$. Post-gravid *age-1(mg44)* F1 homozygotes were at adult day 8–9; F2 homozygotes at day 10; $N2_{DRM}$, *age1(hx546)*, and *daf-16(mu86); age-1(mg44)* double mutants, were all post-gravid adults at adult day 6; and $N2_{DRM}$ dauer larvae 1 day after reaching 98% SDS-resistance. Transcript levels are means of 3 independent biological replicates, normalized to the mean of three control gene (β-actin, T08G5.3, and Y71D11.3) that did not differ significantly among strains.

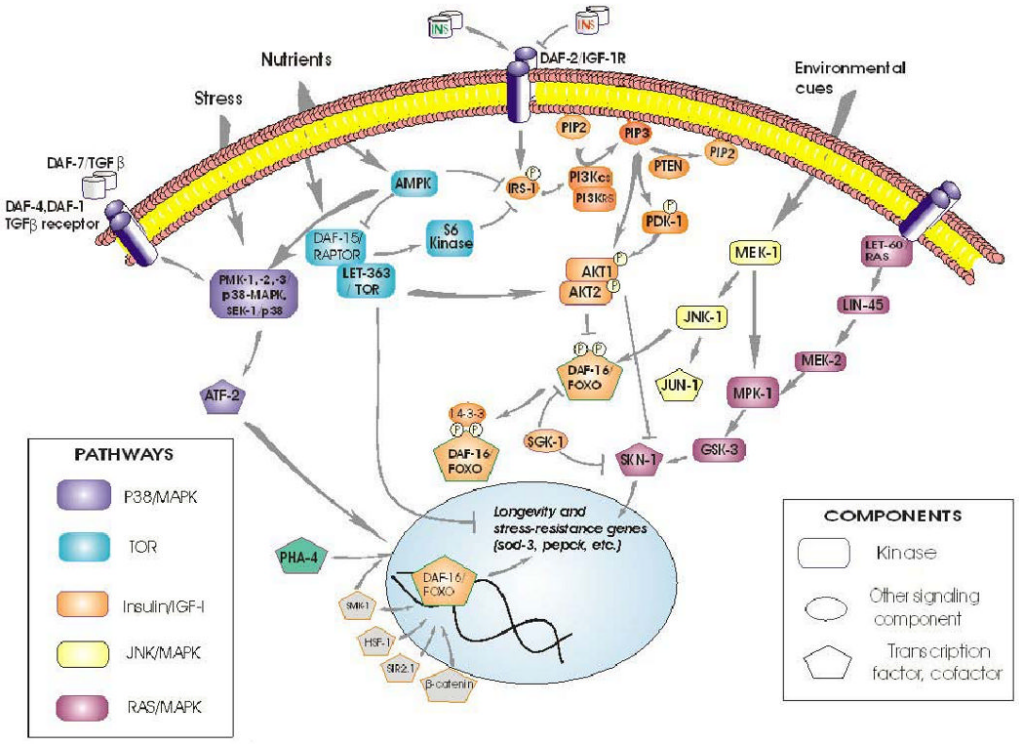

Figure 5. Interactions between IIS and other signal transduction pathways in *C. elegans*

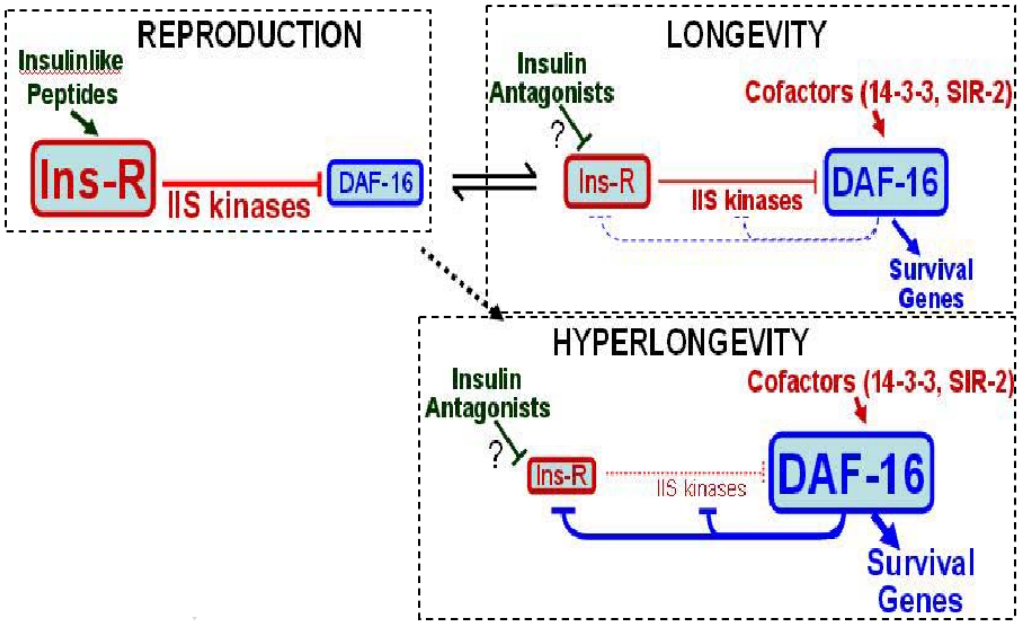

Figure 6. Three system states of a proposed positive-feedback loop

IIS leads through a series of kinase reactions to phosphorylation of DAF-16, sequestering it in the cytoplasm (reproduction mode). If insulinlike antagonists impede IIS, and/or coactivators reinforce DAF-16, the system switches to longevity mode where DAF-16 prevails and transcriptionally represses its own upstream regulatory kinases – promoting dauer formation in development, or life-extension in the adult. This "flip-flop" circuit, with opposing kinase and transcriptional signals, forms a positive-feedback loop. To recover from the dauer state, reproductive kinases must retain partial function, so favorable signals can shift the balance in their direction. Strong *age-1* mutations "fuse the switch" in longevity mode, while conferring a distinctive transcript profile and greatly enhanced survival. Adapted from [30].