Positive Feedback between Transcriptional and Kinase Suppression in Nematodes with Extraordinary Longevity and Stress Resistance

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Abstract

Insulin/IGF-1 signaling (IIS) regulates development and metabolism, and modulates aging, of Caenorhabditis elegans. In nematodes, as in mammals, IIS is understood to operate through a kinase-phosphorylation cascade that inactivates the DAF-16/FOXO transcription factor. Situated at the center of this pathway, phosphatidylinositol 3-kinase 3-kinase (PI3K) phosphorylates PIP2 to form PIP3, a phospholipid required for membrane tethering and activation of many signaling molecules. Nonsense mutants of age-1, the nematode gene encoding the class-I catalytic subunit of PI3K, produce only a truncated protein lacking the kinase domain, and yet confer 10-fold greater longevity on second-generation (F2) homozygotes, and comparable gains in stress resistance. Their F1 parents, like weaker age-1 mutants, are far less robust—implying that maternally contributed trace amounts of PI3K activity or of PIP3 block the extreme age-1 phenotypes. We find that F2-mutant adults have <10% of wild-type kinase activity in vitro and <60% of normal phosphoprotein levels in vivo. Inactivation of PI3K not only disrupts PIP3-dependent kinase signaling, but surprisingly also attenuates transcripts of numerous IIS components, even upstream of PI3K, and those of signaling molecules that cross-talk with IIS. The age-1(mg44) nonsense mutation results, in F2 adults, in changes to kinase profiles and to expression levels of multiple transcripts that distinguish this mutant from F1 age-1 homozygotes, a weaker age-1 mutant, or wild-type adults. Most but not all of those changes are reversed by a second mutation to daf-16, implicating both DAF-16/FOXO-dependent and –independent mechanisms. RNAi, silencing genes that are downregulated in long-lived worms, improves oxidative-stress resistance of wild-type adults. It is therefore plausible that attenuation of those genes in age-1(mg44)-F2 adults contributes to their exceptional survival. IIS in nematodes (and presumably in other species) thus involves transcriptional as well as kinase regulation in a positive-feedback circuit, favoring either survival or reproduction. Hyperlongevity of strong age-1(mg44) mutants may result from their inability to reset this molecular switch to the reproductive mode.

Introduction

IIS depends critically on the presence of PI3

The IIS pathway, governing developmental arrest, metabolism and life span in Caenorhabditis elegans [1–3], is highly conserved from invertebrates to mammals. The single IIS pathway of nematodes corresponds in structure and function to two distinct pathways of mammals that signal metabolic responses to insulin, and growth response to insulin-like growth factor 1 (IGF-1), respectively [4]. IIS disruption was first discovered to enhance longevity in C. elegans [5–8], but it was subsequently shown to also extend life in D. melanogaster and mice [9–11]. Binding of insulin-like peptides to DAF-2, the insulin/IGF-1 receptor of nematodes, modulates receptor autophosphorylation and activation [12]. Active DAF-2 recruits and phosphorylates the AGE-1 catalytic subunit of phosphatidylinositol 3-kinase (PI3K), which in turn phosphorylates the regulatory subunit. Activated AGE-1 then adds a phosphate to phosphatidylinositol 4,5-diphosphate [PI(4,5)P2] at the inositol-3-position, converting it to phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P3 or PIP3].

PI3 plays a dual role in the canonical insulin/IGF-1 pathway. The first pivotal role is membrane tethering of many signaling molecules including AKT-1 and -2, PDK-1, GSK-3 and protein kinase C [13–16]. PIP3-binding recruits or retains many kinases at the cytoplasmic surface of the cell membrane, where these enzymes and their substrates (largely other kinases) are concentrated and, by mass action, interact more efficiently. Because PIP3 quantitatively affects multiple components of the IIS cascade, the
Influence of its concentration is compounded. In addition, PI3K
binding to AKT-1 allosterically exposes a cryptic site recognized by
PKD-1 (phosphatidylinositol-dependent kinase 1), allowing
AKT phosphorylation and activation [17]. In this second role, PI3K
may act catalytically, in that a single molecule of PI3K has the
potential to bind successively to many AKT-1 molecules, enabling
their activation. Although AKT-1 is the only target for which this
allosteric role has been documented [17], it is possible that other
signaling molecules that also possess high-affinity PI3K binding
sites (termed “Pleckstrin homology domains”) may be similarly
controlled. In any event, we infer that insulinklike signaling should
be exquisitely sensitive to PI3K depletion, and that AKT-1 action
which extends far beyond IIS [18,19]) may be absolutely
dependent on the presence of at least trace amounts of PI3K.
The AKT-1/AKT-2/SGK-1 complex, once all of its constitu-
ent kinases have been activated by PKD-1 [20], phosphorylates the
DAF-16/FOXO transcription factor at sites that block its entry into the
nucleus, where it would activate or repress transcription of hundreds of target genes, including many that
di modulus metabolism, reproduction, life span, and resistance to
oxidative stresses [21–24].

IIS mutations have wide-ranging effects on longevity

Reduction-of-function mutations impairing the C. elegans IIS
pathway (e.g., daf-2 and age-1 mutations) cause these worms to
arrest development as dauer (alternative stage-3) larvae [1–3]. If
allowed to mature at a permissive temperature, temperature-
sensitive (ts) daf-2 mutant adults can attain twice the normal
longevity [6]; life extension ranges from 1.1- to 2.5-fold for
different daf-2 alleles [25]. A ts mutant allele of age-1, hs546, was
discovered by Klass [26] and reported to confer 40% and 65% life
extension at 20° and 25°C respectively [5,27,28]. Two constitutive
age-1 alleles, m333 and mg44, were initially reported to extend C.
elengans life span by 2- to 2.6-fold [23,29]; these survivals were
conducted only for first-generation (“F1”) homozygotes. We
recently observed that second-generation age-1(mg44) and (m333)
larvae slowly mature at 15–20°C into adults that live close to ten
times as long as near-isogenic wild-type controls, and are highly
resistant to oxidative and electrophilic stresses [30]. These
exceptional worms have mean and maximal adult life spans at
least three times those conferred by any other longevity-extending
mutation, and throughout their adult lives they appear and behave
very much like wild-type worms of a tenth their age. Addition of a
second mutation in the daf-16 gene largely or entirely reverses life-
span extension and other phenotypes of all daf-2 or age-1 mutations
examined to date [3,6,29,30].

Studies of IIS-pathway mutants in C. elegans and other taxa have
provided valuable insights into genetic mechanisms regulating life
span [4]. The molecular basis for the extreme survival phenotypes
of age-1(mg44) F2 homozygotes remains unknown, and cannot be
assumed to differ only in degree from molecular mechanisms that
underlie 4- to 5-fold lesser life extensions seen in other IIS mutants.
The key may be PI3K, which plays both structural and catalytic
roles in signal transduction [17,31], and is thought to mediate both
DAF-16-dependent and -independent signaling [32]. Strong age-1
mutants, lacking all class I PI3K activity, have no direct route to
produce PI(3,4,5)P3 [31]. As a result, they are expected to be deficient in
all enzyme activities that require PI3K, either for activation by
regulatory kinases, or for membrane tethering which ensures proximity of kinases to their targets [17,31].

In the present study, we sought evidence to support such a
broad role of PI3K in the unique properties of age-1(mg44)-F2
adults. This role is an inferred one, since even normal PI3K levels
(in unstarved N2 worms) are too low for detection by existing
methods; detectable levels are attained in starved, peroxide-
stressed wild-type worms but not in similarly stressed age-1-mutants
[33]. We were able to document the expected widespread
disruption of protein kinase activity in age-1(mg44)-F2 worms,
while making the unexpected observation that the same kinases
are chiefly inhibited at the transcriptional level. Direct measure-
ment of transcripts confirms silencing of kinase gene expression,
leading us to propose a novel “hybrid” positive-feedback loop in
which the IIS kinase cascade that inhibits the DAF-16/FOXO
transcription factor, is itself attenuated by DAF-16-mediated
transcriptional silencing of upstream kinases.

Results

Very long-lived age-1 mutant worms are broadly
deficient in protein kinase activity

The age-1(mg44) kinase-null mutants should be deficient in
phosphatidylinositol 3,4,5-triphosphate production. Given the
importance of the PI3K molecule in signal transduction events
originating from many membrane-receptor kinases, we anticipated
that phosphorylation of numerous proteins may be impaired in
those mutants. To initially assess the breadth of this impairment,
we compared in vitro kinase activities with respect to endogenous
substrates for five age-1 mutant strains, each normalized to a wild-
type N2DRM stock (Figure 1A–1C).

Panels A and B illustrate a typical experiment, and panel C
summarizes results for replicate experiments with independent
expansions of each group. The first-discovered and most widely
used age-1 allele, hs546 [5,27,28], showed 32% less kinase activity
than N2DRM (Figure 1C). However, worms bearing the age-
1(mg44) allele had less than 10% of wild-type kinase activity,
whether maternally protected first-generation (F1) or very long-
lived second-generation (F2) homozygotes. The F2 worms had
somewhat lower kinase activity than F1 (7.3 vs. 8.6% of N2DRM,
P = 0.05), although the difference was consistently much greater
for specific bands (see Figure 1B). Staining of total protein showed
similar loads for all samples, although handing patterns differed (Figure 1A). One obvious difference between age-1(mg44) and the other strains is that these mutants are totally infertile in the F2 generation, despite the presence of syncytial nuclei [30]. Several controls exclude this as an explanation for the mutants' lack of kinase activity. age-1(mg44) F1’s have similar kinase levels when gravid (day 2 of adulthood) or post-gravid (adult day 6; see Figure S1). Moreover, N2DRM eggs contain about half as much kinase activity as their parents, per weight of protein (Figure S1), so their absence would not reduce kinase activity in any case. Deficiency of kinase activity is not a characteristic of dauer larvae, which exhibit absence would not reduce kinase activity in any case. Deficiency of kinase activity as their parents, per weight of protein (Figure S1), so their absence would not reduce kinase activity in any case. 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The consequences of adding a daf-16 mutation are quite different for the two age-1 alleles: in age-1(hx546) worms, the daf-16(m26) mutation more than doubled the in vitro kinase activity, from 68% of wild-type to 160%, whereas this mutation restored less than half of the kinase deficiency due to the age-1(mg44) allele. Insofar as kinase suppression is reversed in daf-16; age-1(mg44) double mutants, we infer that activity is inhibited in part through the DAF-16/FOXO transcription factor. However, reversion is far from complete, by either the m26 (point-mutant) or the mu86 (large-deletion) allele of daf-16 (see Figure 1C and Figure S1). This implies that a large proportion of observed kinase silencing is DAF-16-independent—perhaps reflecting direct effects of PIP3 depletion on kinases other than AKT, or AKT targets other than DAF-16/FOXO.

To corroborate low protein-kinase activity of age-1(mg44) adults, and to distinguish whether they are deficient for many protein kinases or a few very active ones, we constructed arrays of 70 synthetic peptides comprising 50 near-consensus kinase sites from the C. elegans proteome and 20 from mouse or human proteins. Phosphorylation in vitro was observed on 29 peptides, representing potential substrates for at least 18 distinct kinases (Figure S2 and Table S1). Protein kinase activity in extracts from age-1(mg44) F2 adults was reduced by 1.8- to >8-fold, relative to isogenic N2DRM postgravid worms (each at nominal P<0.05), for 22 of the 29 kinase targets that were phosphorylated in vitro. Addition of the daf-16(mu86) mutation produced essentially complete reversion, or hyper-reversion (activity>N2DRM), for 17 of those 22 peptides.

In view of the reduced protein-kinase activity of age-1(mg44) worms, we anticipated that their steady-state level of protein phosphorylation would also be depressed. To assess this, phosphoproteins were separated by acrylamide gel electrophoresis and compared among wild-type and age-1-mutant strains of C. elegans (Figure 1D–1F). Total protein staining (panel D) demonstrated even loading, while panel E shows the same gel stained with Pro-Q Diamond to detect and quantify phosphoproteins. Results for three replicates (independent expansions of each strain) are summarized in panel F. Relative to wild-type N2DRM, age-1(mg44) worms had ~16% less phosphoprotein staining (marginally
significant at $P<0.05$), while age-1(mg44) homozygous F2 adults showed a 41% reduction in steady-state phosphoprotein level ($P<0.001$). The daf-16(m26) mutation restores either allele to ~92% of the N2DRM level. This finding is also supported by 2-D dual-fluor phosphoprotein gels (Figure 2), in which 72% of the phosphoprotein spots resolved (1199/1669) were reduced at least twofold in F2 age-1(mg44) adults relative to N2DRM. The deficiency of total phosphoprotein content is less pronounced than that of protein kinase activity, in age-1(mg44)-homozygous F2 adults, which is not surprising given that phosphoprotein levels reflect the steady state, i.e., a balance between kinase and phosphatase activities. We present evidence (next section) that the PTEN phosphatase is indeed downregulated in age-1(mg44).

**Transcriptional suppression of signal-transduction genes in age-1(mg44) worms**

F2 homozygotes for age-1(mg44) are expected to produce only truncated class-I PI3Kcs, lacking the kinase domain and C-terminus of the protein. These worms indeed lack the main bands recognized by antibodies to the AGE-1 C-terminal region (Figure S3); residual bands may represent class-II and -III homologs of AGE-1. PIP3, formed exclusively by class-I PI3K, should thus be greatly reduced or absent. PIP3 is strictly required for PDK-1 activation of AKT kinase, which then phosphorylates and inactivates DAF-16/FOXO. Kinases that require PIP3 binding for membrane tethering or kinase activation [17,31], such as AKT, PDK-1, and SGK-1, are expected to show marked suppression of activity, which cannot be directly reverted by a daf-16 mutation. The surprising observation that mutations to daf-16 restore nearly half of the age-1(mg44)-F2 kinase deficiency, and >70% of its phosphoprotein deficit, implies that their inhibition must be mediated in part by DAF-16/FOXO. Such regulation could be direct (DAF-16 suppresses transcription of many kinase genes) or indirect (DAF-16 suppresses one or a few kinases, or stimulates one or a few phosphatases, which then suppress other kinases by impeding or opposing their phosphorylation). To test direct effects of DAF-16/FOXO, we used real-time polymerase chain reaction (RT-PCR) to quantify the effects of age-1 alleles, with or without added inactivation of daf-16, on transcript levels for IIS genes and a panel of other signaling components, representing a wide range of transduction pathways.

**IIS genes.** We first assessed genes involved in insulin-like signaling. Results of a comparison of independent biological replicates for each strain are shown graphically in Figure 3, and data for these and additional genes are tabulated (including statistical significance) in Table 1. Transcript levels in age-1(mg44) F2 adults were found to be markedly reduced, by factors of 4- to 14-fold, for all kinase genes of the IIS pathway except akt-1

![Figure 2. Most phosphoproteins are depleted in age-1(mg44) F2 adult worms.](image-url)
This silencing extends even to the *age-1* gene itself (encoding the class-I catalytic subunit of PI3K) and, to a lesser extent, the class-II and class-III PI3K CS genes (Table 1). Three genes encoding transcriptional cofactors of DAF-16/FOXO were significantly inhibited in *age-1(mg44)*: *smk-1* [34] and *sir-2.1* [35], encoding coactivators that reinforce unphosphorylated DAF-16/FOXO, and also *par-5*, encoding a member of the "14-3-3" family that binds and inactivates IIS-phosphorylated DAF-16/FOXO [36]. In addition, the *daf-18* gene encoding PTEN phosphatase (opposing PI3K) [37,38] was attenuated 12-fold.

The *C. elegans* genome contains at least 38 genes encoding putative insulinlike peptides (ILPs), although functional roles in the initiation of IIS have been established for rather few ILPs [12]. Genes encoding six ILPs, known or presumed ligands of the DAF-2 insulin/IGF-1 receptor, were selected for study based on prior evidence of a biological function, as cited in WormBase (www.wormbase.org). Five of these (including genes for known IIS antagonists INS-1 and INS-18) were upregulated 3- to 10-fold in *age-1(mg44)* F2 adults, whereas the *ins-7* ILP gene—an established ILP-receptor agonist for which RNA interference (RNAi) extends life [39]—had roughly half the wild-type transcript level. In addition, two positive targets known to be induced by DAF-16/FOXO [24,40] serve to confirm its enhanced activity in *age-1(mg44)* adults: *sod-3*, encoding mitochondrial superoxide dismutase-3 (up 8.6×), and R11A5.4, which encodes phosphoenolpyruvate carboxykinase (PEPCK), a key activator of gluconeogenesis (up 8.5×).

For the most part, this profile of IIS transcripts is consistent with concerted silencing of the IIS pathway in *age-1(mg44)*, leaving DAF-16 in its active state, unphosphorylated at sites that would exclude it from the nucleus. Thus, in *age-1(mg44)* F2 adults, insulinlike peptide genes that antagonize IIS are upregulated while...
Table 1. Expression of signal-transduction genes in *C. elegans* strains, assessed by real-time polymerase chain reaction.

| Pathway                  | Gene       | Protein Function / Notes                           | DAF-16 sites | hx546 | daf-16; hx546 | F1 mg44 | F2 mg44 | daf-16; mg44 | dauers |
|--------------------------|------------|---------------------------------------------------|--------------|-------|--------------|---------|---------|-------------|--------|
| **Insulin/IGF-1 Signaling (IIS) pathway** |            |                                                   |              |       |              |         |         |             |        |
|                          | ins-1      | DAF-2/IIS antagonist                               | 1, 1         |       | 16.0*        | 4.0     |         | 10.0***     | 3.5    | 80.0****    |
|                          | ins-5      | neuronal expression; no effect of RNAi            | 1, 3         | 1.9  | 0.90         | 24.0**  | 4.5*    | 1.0         | 7.4    |
|                          | ins-6      | poss. agonist; no effect of RNAi                   | 0, 2         | 1.9  | 1.4          | 10.7**  | 3.2*    | 1.2         | 2.2    |
|                          | ins-7      | DAF-2/IIS agonist; RNAi extends life               | 0, 2         |       | 5.3*         | 5.3*    | 0.95    | 0.55        | 1.2    | 1.6         |
|                          | ins-14     | possible DAF-2 / IIS agonist                      | 0, 0         | 1.5  | 1.1          | 26.7**  | 6.8**** | 1.8*        | 22.0****|
|                          | ins-18     | IIS antagonist; structure similar to INS-1         | 1, 1         | 1.4  | 1.5          | 3.0     | 5.0***  | 1.1         | 16.1****|
|                          | daf-2      | insulin/IGF-1 receptor                             | 1, 2         |       | 0.38*        | 0.90    | 1.6     | 0.20******  | 0.19****** | 0.55    |
|                          | ist-1      | insulin-receptor substrate 1 (IRS-1)              | 3, 0         | 2.0  | 0.55         |         | 1.3     | 1.8*        | 0.90   | 6.5         |
|                          | age-1      | PI3Kcs (class-I)                                  | 0, 0         | 1.6  | 0.80         | 0.06*** | 0.07**** | 0.63        | 0.17**   |
|                          | F39B1.1    | PI3Kcs (class-II)                                 |              | 0.90 | 0.80         | 0.25*** | 0.90    | 0.90        |        |
|                          | vps-34     | PI3Kcs (class-III); vesicular trafficking         | 0, 2         | 1.7  | 0.90         | 0.37**  | 1.0     | 0.44        |        |
|                          | daf-18/pten | PIP3 phosphatase (opposes AGE-1)                  | 0, 0         | 1.8  | 1.0          | 0.35    | 0.08**** | 1.0         | 0.03****  |
|                          | sgk-1      | serum/glucocorticoid-dependent kinase 1           |              | 1.0  | 0.80         | 0.18*   | 0.25**  | 0.85        | 0.15*    |
|                          | pdk-1      | phosphoinositide-dependent kinase 1               | 2, 1         | 0.44 | 0.63         | 1.4     | 0.18**  | 0.52        | 0.50     |
|                          | akt-1      | ortholog of S/T kinase AKT/PKB (RAC-a)           | 3, 1         | 1.0  | 1.0          | 1.3     | 0.60    | 1.2         | 0.46     |
|                          | akt-2      | homolog of S/T kinase AKT/PKB (RAC-c)            |              | 1.9  | 0.64         | 5.2     | 2.6     | 0.50        | 3.2      |
|                          | daf-16     | FOXO1 / FOXO3 forkhead transcrip't factor         | 3, 3         | 0.90 | 0.40         | 0.70    | 0.60*   | 0.50        | 1.0      |
|                          | sod-3      | Mn-superoxide dismutase (mitoch. SOD)             |              | 0.57 | 0.55         | 3.0     | 9.0***  | 0.90        | 7.0*     |
|                          | pepck      | phosphoenolpyruvate carboxykinase                 | 0, 1         | 0.90 | 1.1          | 0.80    | 8.5*    | 0.95        | 0.90     |
| **DAF-16-interacting transcriptional regulators** |            |                                                   |              |       |              |         |         |             |        |
|                          | cst-1      | DAF-16 kinase, responds to oxidat.-stress         | 0, 0         | 2.3  | 0.90         | 1.3     | 1.0     | 1.2         | 2.6*     |
|                          | bar-1      | β-catenin transcrip't factor (Wnt pathway)        | 0, 3         | 0.60 | 1.1          |         | 0.60    | 0.70        |         |
|                          | smk-1      | transcrip't coactivator of DAF-16 and PHA-4      | 1, 3         | 0.72 | 1.1          | 0.70    | 0.28**  | 0.87        | 0.18***   |
|                          | par-5      | 14-3-3 family; sequesters pDAF-16                 | 1, 3         | 1.5  | 1.1          | 0.48    | 0.28**  | 1.1         | 0.13***   |
|                          | ftt-2      | 14-3-3 family; sequesters pDAF-16                 |              | 1.5  | 0.90         | 0.62    | 0.86    | 1.0         | 0.63     |
|                          | sir-2.1    | transcriptional coactivator of DAF-16             | 0, 1         | 0.91 | 1.1          | 0.95    | 0.26**  | 0.89        | 0.29**    |
| **TGF-β signaling**      |            |                                                   |              |       |              |         |         |             |        |
|                          | daf-7      | TGF-β family member (ligand, agonist)             |              | 7.0* | 2.6          | 3.0     | 5.3**   | 1.2         | 19.7**   |
|                          | daf-1      | TGF-β family receptor type I                      | 1, 0         | 2.2  | 1.5*         | 0.50    | 0.22**** | 1.6         | 1.1      |
|                          | daf-4      | TGF-β family receptor type II                     | 1, 3         | 1.5  | 1.0          | 0.60    | 0.24**** | 1.5         | 1.2      |
|                          | daf-12     | VDR homolog, controlled by TGF-β, IIS             |              | 1.6  | 1.0          | 0.70    | 1.6     | 0.80        | 3.1*     |
|                          | daf-3      | SMAD transcription factor                        | 2, 1         | 1.5  | 0.90         | 0.30*** | 0.30**  | 1.9**       | 1.7*     |

* TGF-beta signaling outputs to p38 and ERK MAPK pathways
ins-7 (the only confirmed IIS agonist) is downregulated. Genes
supplying the IIS kinase cascade that inactivates DAF-16/FOXO
(daf-2, age-1, skg-1, and pdk-1) are all downregulated, whereas ist-1,
encoding a homolog of mammalian IRS-1 and IRS-2 [41], which
serve as convergence points for many inhibitory inputs from other
signaling pathways [41,42], is upregulated. Moreover, aak-2, the
sole AMP-dependent kinase (AMPK) gene implicated in DAF-16/
FOXO activation [43,44], and shown to extend lifespan when
over-expressed [45], is upregulated in age-1(mg44) F2 adults
(Table 1). Apparent exceptions include the low transcript levels
for two genes that antagonize IIS: daf-18, encoding the PI3-
phosphatase PTEN, and par-5, encoding one of several 14-3-3
proteins that sequester IIS-phosphorylated DAF-16—genes that
arguably are superfluous in the absence of PIP3 and IIS-mediated
suppression of kinase activities (Figure 1), we extended the

### Table 1. Cont.

| Pathway | Gene | Protein Function / Notes | DAF-16 sites<sup>1</sup> | hx546 | daf-16; hx546 | F1 mg44 | F2 mg44 | daf-16; mg44 | dauers |
|---------|------|-------------------------|------------------------|-------|--------------|--------|--------|-------------|-------|
| AMPK/TOR pathway (nutrient sensing) | aak-1 | AMP-dependent kinase 1 | 2, 1 | 1.2 | 0.80 | 0.47 | **0.17*** | 1.0 | **0.05*** |
|         | aak-2 | AMP-dep'. kinase 2 (activates DAF-16) | -- | **1.7** | **1.5*** | **0.60** | **2.2** | **0.57** | **6.4*** |
|         | let-363 | FRAP/mTOR ortholog: part of PIK1, 0 family | | 0.70 | 0.70 | 1.0 | **0.25** | 0.60 | 0.47 |
|         | daf-15 | ortholog of RAPTOR, mTOR reg. 2, 0 subunit | | 0.80 | 0.90 | 0.80 | **0.15*** | 0.80 | **0.22** |
|         | rsk-1 | SKCa ribosomal S6 protein kinase 1, 0 | 1 | 1.6 | 0.90 | 1.0 | 1.5 | 1.2 | **3.9*** |
| p38-MAPK (stress response, immune response) | pmk-1 | p38 MAPK 1 (mitogen activated 3, 0 prot. kinase) | -- | 1.0 | **3.8** | 0.60 | 0.70 | 1.0 |
|         | pmk-2 | p38 MAPK 2 (mitogen activated 2, 0 prot. kinase) | -- | **0.20*** | 0.40 | **0.20*** | 0.18 | **0.50*** |
|         | pmk-3 | p38 MAPK 3 (mitogen activated 2, 0 prot. kinase) | -- | 0.40 | 1.2 | 0.56 | **0.20*** | 1.5 |
|         | atf-2 | cAMP-dep. transcription factor family (+) | 0, 1 | 2.5 | 1.1 | **44.1*** | 3.9 | **2.6** | 9.6*** |
| JNK-MAPK (immune signaling, stress response) | jnk-1 | Jun N-terminal kinase 1 | 0, 0 | 1.2 | 1.4 | 0.95 | **3.5*** | 1.2 | 13.2***** |
|         | jun-1 | JUN subunit, AP-1 transcription factor (+) | 2, 2 | 0.74 | 1.0 | 1.1 | **0.6** | 0.95 | **2.2*** |
| ERK-MAPK (growth-factor and immune responses) | let-60 RAS | small membrane GTPase co-receptor | 0, 0 | 0.50 | 0.30 | **0.18*** | **0.16*** | 0.50 | 0.30 |
|         | lin-4S | Ser/Thr kinase of MEK-2, activ byO, 1 LET-60 | 1.0 | 0.80 | 1.1 | **0.20** | 0.90 | **0.33** |
|         | mek-1 | stress-response ERK for JNK-1, MPK-1 | 3, 1 | 0.75 | 0.50 | **0.30** | **0.23**** | **0.38** | 0.70**** |
|         | mpk-1 | MAPK, transduces develop'l RAS 0, 2 signals | 0.80 | 1.8 | 2.1 | **0.23*** | 1.0 | **0.21*** |
|         | gsk-3 | glycogen-synthase kinase 3 | 0, 0 | 0.56 | 0.50 | **0.28** | **0.20*** | 0.90 | **0.13*** |
|         | skn-1 | oxid-damage-response TF, inhib'd by IIS | 0, 0 | 1.3 | 1.0 | 0.55 | **0.30*** | 1.6 | 0.60 |

Key: –, not assessed. Significance of difference from N2DRM (by 2-tailed t-test): *, nominally significant at P<0.05.
**P<0.01; ***P<0.001; ****P<1E–4; *****P<1E–5; ******P<1E–6.
<sup>1</sup>Number of exact consensus DAF-16 sites in upstream 5-kb span, (a, b), where a=GTAAA(C/A)A, and b=CTATACA.

For each gene tested, wild-type N2DRM adults were compared to four age-1 mutant populations and to dauer larvae. The age-1(mg44) worms are F1 homozygotes at day 8–9 of adulthood, by which time they were post-gravid, or F2 homozygotes at day 10 (18 days post-hatch); other groups (N2DRM, age-1(hx546), and daf-16(mu86); age-1(mg44) double mutants) were harvested when post-gravid (days 6–8 of adulthood), or as dauer larvae (N2DRM only) from starved, dense cultures 1 day after >98% of worms had become resistant to lysis by 1% sodium dodecyl sulfate. Transcript levels were assayed for 3–8 independent biological replicates, with two cDNA syntheses and RT-PCRs for each. Numbers shown are transcript ratios for each group indicated (in the column header) relative to transcript levels of the same genes in near-isogenic N2DRM controls. All C(t) data (threshold cycle numbers) were normalized to the mean values for three control genes (β-actin, T08G5.3, and Y71D11.3) that did not change among the strains/groups tested. Changes that were at least nominally significant (P<0.05) are emphasized with bold font.

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than 3-fold. In contrast, transcripts are elevated for the daf-7 gene, encoding one of four known TGF-β peptides in C. elegans. The nutrient-responsive AMPK/TOR pathway is largely silenced, although its translational output, rks-1/S6K, is not. The two AMPK genes, akt-1 and akt-2, clearly serve distinct functions (in view of differing RNAi effects), and may diverge in their roles with respect to inhibition of the TOR complex and IIS (via IS1-1/IRS-1/2), or activating phosphorylation of DAF-16/FOXO [43–45]. The p38/MAPK pathway, which also interacts with IIS [47], is ambiguous in that only one of the three p38/MAPK genes is significantly downregulated (pmk-2, down 5-fold, \( P<0.005 \)), while the other two are much less affected and af-2, mediating transcriptional output from this pathway, is induced (Table 1). A similar conflict of effects is evident in the JNK/MAPK pathway, wherein jnk-1 (encoding Jun N-terminal kinase 1) is induced in age-1(mg44) F2 adults, but one of its targets, jun-1 encoding the JUN transcription factor, is modestly downregulated. This suggests that the critical activation target of JNK-1 (in the context of age-1 longevity) is not JUN, but DAF-16/ FOXO (see also [48]).

Altogether, this targeted gene survey indicates widespread silencing of signal-transduction genes in age-1(mg44) F2 adults, with significant declines relative to N2DRM (\( P<0.01 \)) for 23 of the 47 genes tested, and increases for 12 genes. The declines imply attenuation of IIS, TGF-β, and AMPK/MAPK pathways, and either silencing or redirection of the AMPK/TOR nutrient-sensing pathway. Two pathways implicated in immune and stress responses, p38/MAPK and JNK/MAPK, do not appear to have all outputs inhibited at the transcript level. The bias toward inhibition over induction cannot be a reflection of global transcriptional silencing, because all data were normalized to the mean of three control genes (β-actin, T08G5.3, and Y171D11.3) that did not change among the strains tested. However, the bias is consistent with the results of a microarray survey (Ayaddevara et al.; submitted) which indicated that differential gene expression in strong age-1 mutants reflects primarily transcriptional suppression targeting a few hundred genes, largely or entirely specific to those alleles.

**Expression changes in age-1(mg44)** F2 adults do not simply recapitulate a younger adult state in wild-type worms. Might the observed differences in gene expression reflect the considerable differences in physiological age (or in chronological age) among the young-adult worms being compared? Gene expression in Drosophila lines, varying in longevity by up to threefold, was reported to fall into two classes: some genes alter transcript levels over time, independent of lifespan, while others show expression patterns that scale with lifespan [49]. Of the genes that appeared strongly modulated in age-1(mg44) F2 adults, a subset was chosen to represent four signaling pathways, and assessed in wild-type N2DRM adults at several adult ages, as well as one or two ages of age-1(mg44) F2 adults. This time series (Figure 4) demonstrates that only a few of the expression changes observed in very long-lived age-1 worms could be interpreted as reflections of their physiologically youthfull state relative to wild-type worms. In particular, expression in age-1(mg44) F2 worms at 8 and 16 days of adult age (5 and 10% of their mean adult life spans) did not overlap the levels seen in N2DRM worms at 1, 3 or 6 days adult age (6, 18 or 37% of life span), for the genes ins-1, ins-14, daf-16, pcpk, daf-1, daf-4, akk-2, or let-363. Thus, the differences in their expression mirror genotype rather than aging. In contrast, scaling to lifespan largely eliminates the difference between strains for two genes of the ERK/MAPK pathway (daf-15 and nku-1), while intermediate outcomes were seen for pdk-1, daf-3, and tin-45.

**Silencing involves both daf-16-dependent and –independent routes.** Of 35 nominally significant (\( P<0.01 \)) transcriptional differences between age-1(mg44) F2 and N2DRM adults, 25 were completely reversed (to within 20% of the N2DRM level, or over-reverted) by mutations inactivating daf-16; a further 9 genes showed partial reversal, while two, daf-2 and pmk-2, did not revert at all. Four instances of partial or negligible reversal (ins-14, daf-2, pmk-2 and pmk-3) entail expression levels for daf-16(mu66); age-1(mg44) adults that differed significantly from N2DRM (each \( P<0.01 \); combined \( P<10^{-5} \)). Results were similar for the mu26 and mu66 alleles of daf-16; Table 1 shows only data for the mu66 allele in which most daf-16 exons are deleted. These data demonstrate that age-1(mg44) F2 effects on transcription are primarily mediated by DAF-16/FOXO, but are also supplemented by DAF-16-independent mechanisms—paralleling the conclusions drawn from earlier data on daf-2 mutants [50].

**Expression changes differ between F2 and F1 age-1(mg44)** homozygotes. Transcriptional modulation is also seen in first-generation (F1) age-1(mg44) adults, for which inductions occasionally exceeded the level seen at F2. In fact, the three highest induction factors were observed in F1 adults, for ins-5 (24×), ins-14 (27×), and af-2 (44×), with ins-6 (11×) also showing substantially higher transcription levels in F1 worms than in F2. However, expression of 21 genes was altered 2- to 8-fold more in F2 worms than in F1 (e.g., ist-1, vps-34, daf-18, sod-3, smk-1, sir-2.1, aak-2 and daf-15), while 6 genes were roughly equal at the two generations (age-1, sgk-1, daf-3, let-60, mek-1, and gsk-3). Clearly, the consequences of maternal protection differ among these genes.

**Comparison to other IIS mutants and to dauer larvae.** F2 age-1(mg44) adults generally show far more pronounced transcriptional effects than the weaker age-1 allele, hs546 (Figure 3 and Table 1). Only two of 47 tested genes (4%) changed significantly (at \( P<0.01 \)) in age-1(hs546) adults: ins-1 (up 16× over N2DRM) and ins-7 (up 5.3× in hs546, but down 2-fold in mg44-F2 adults). This is in stark contrast to 35 significantly differential genes (74%) seen in mg44-F2 adults—underscoreing the quite atypical properties of the stronger age-1 allele. The widely studied daf-2(e1370) strain was tested for transcription levels of 17 genes, of which only 4 (24%) differed significantly from N2DRM (at \( P<0.01 \); see Table S2). These four genes (ins-1, sgk-1, daf-7 and aak-2) were all upregulated in the daf-2 mutant. Like age-1(hs546), daf-2(e1370) is a temperature-sensitive mutant of much weaker effect than the age-1 nonsense mutants; both give a life extension of about twofold in our hands, after outcrossing into the N2DRM background (data not shown). Both daf-2(e1370) and age-1(hs546) are upregulated for most signaling components tested in the IIS and TGF-β pathways, whereas nearly all of these genes (all except ins-1, encoding an IIS antagonist, and daf-7, encoding one of four TGF-β ligands) are strongly suppressed in age-1(mg44) F1 and especially F2 adults (Table S2).

F2 age-1(mg44) adults differed significantly from dauer larvae for 43% (20/46) of the signal-transduction genes tested for both; the same number (20) had transcript levels that were within twofold of those in dauer larvae (Table 1)—suggesting that these two very long-lived populations achieve resistance to aging through distinct but partially overlapping gene-expression strategies.

The transcript-level changes observed in age-1(mg44) F2 adults were cross-checked against those previously reported for either daf-2(e1370) adults, wild-type dauer larvae, or both [21,24,31–33]. Few commonalities were found between the present data and these gene lists, which comprise the most highly and significantly altered genes in microarray surveys. The differences may in part reflect the greater sensitivity of RT-PCR to detect and quantify low-abundance transcripts; however, data in Tables 1 and Table S2 imply that transcriptional shifts for age-1(mg44) F2 adults differ both qualitatively and quantitatively from those seen in other mutants.
Functional consequences of transcriptional suppression in *age-1(mg44)* worms

F2 *age-1(mg44)* adults, which are 4- to 5-fold longer-lived than F1 adults (comparing data of [8,29] to [30]), also outperform their parents with respect to resistance to oxidative and electrophilic stresses (Figure 5A and 5B). Relative to N2DRM controls, survival in 5% hydrogen peroxide is extended 2-fold in F1 adults, but 10-fold in their F2 progeny. Because *age-1(mg44)*-F2 adults barely reached 20% mortality after 24 h, by which time 100% of worms had died in all other groups, survival time is here compared at a threshold of 20% mortality. Survival of an electrophilic stress, 4-HNE (similarly defined as time to 20% mortality) increased 1.6-fold in *age-1(mg44)* F1 worms but 5-fold at F2, with reference to N2DRM. Although resistance to these stresses is restored almost to wild-type levels in double mutants with *daf-16*, we note that reversion is not quite complete, whether using the weaker *daf-16(m26)* allele [30] or the *daf-16(mu86)* deletion allele (Figure 5A), indicating that such stress-resistance traits are mediated in part by a DAF-16-independent pathway. These results parallel the incomplete reversion, in *daf-16; age-1(mg44)* double mutants, seen for in vitro kinase activity and phosphoprotein levels (Figure 1) and for transcript levels of several genes (Table 1).

Most or all of the *age-1(mg44)*-downregulated genes are essential for nematode growth and development. That is, double-stranded RNAs (dsRNAs) targeting them, administered to developing *C. elegans*, produce embryonic lethality or larval arrest [54–56]. The impact of such knockdown, however, has thus far remained largely unstated in adults. Because resistance to oxidative stresses is a


common feature of many long-lived \textit{C. elegans} mutants [57], and in particular parallels longevity in the \textit{age-1} allele set studied here (Figure 5 and [30]), we employed it as a short-term assay to evaluate the contribution of individual-gene downregulation, to the exceptional survival of \textit{age-1(mg44)-F2} adults in both benign and toxic environments.

Hydrogen peroxide resistance was measured in duplicate experiments, for wild-type \textit{N2DRM} worms that had been exposed to dsRNA-expressing bacteria targeting 10 genes for which transcript levels are markedly reduced in \textit{age-1(mg44)-F2} adults. Genes were selected from among those not directly involved in the IIS pathway, but representing a variety of other signaling pathways, and for which RNAi constructs were available from the Ahringer library [56]. \textit{E. coli}, either harboring an empty-vector control or expressing one of 10 gene-targeted dsRNA species, were fed to mature adults (days 3 through 6 after the \textit{L4/adult} molt) so as to preclude effects on development. Survival curves, during subsequent exposure to 5-mM \textit{H$_2$O$_2$}, are shown in Figure 5C. RNAi for four of the ten genes (encoding a transcription factor and three components of distinct protein-kinase signaling cascades) produced highly significant gains in peroxide survival (each $P<0.001$), and a fifth dsRNA exposure offered marginally significant protection ($p=0.03$). The remaining five dsRNA treatments had no discernible effect on survival, compared to worms exposed only to the empty expression vector. The above results were reproduced in an independent experiment, with the same four genes attaining $P<0.001$, while \textit{vps-34} achieved $P<0.08$.

Genes (and encoded proteins) for which RNAi knock-down conferred a protective effect were \textit{daf-3} (SMAD transcription factor) and \textit{daf-4} (TGF-$\beta$ receptor, a Ser/Thr kinase), both involved in TGF-$\beta$ signaling; \textit{aak-1} (AMP-dependent protein kinase 1), part of the AMPK/TOR pathway; \textit{let-60} (RAS-family GTPase activating MAPK), part of the ERK-MAPK pathway, and \textit{vps-34} (class-III PI3KC, involved in vesicular trafficking and autophagy). None of these individual RNAi effects matched the peroxide survival of untreated \textit{age-1(mg44)} F2 adults at 62 days of adult age (large diamond symbols, Figure 5C). These data demonstrate that transcript-level changes seen in \textit{age-1(mg44)-F2} homozygotes favor oxidative-stress survival. They may also contribute incrementally to the greatly enhanced longevity of \textit{F2} homozygotes, but we have not been able to confirm such effects. When begun at the end of larval development, RNAi to \textit{ak-1} and \textit{let-60} extended survival by 7–11\%; while \textit{let-60} dsRNA reduced it by ~12\% (data not shown). Such small effects on life span require large groups to reach significance; moreover, significance in one experiment provides little assurance that independent replicates will attain significance. This may reflect the low statistical power inherent to survivals of modest size, and/or inability to control environmental variance among experiments.

**Discussion**

First-generation homozygotes for the \textit{age-1(mg44)} mutation develop normally into fertile adults at 15–25$^\circ$C, and display stress resistance and life extension typical of many IIS mutants [8,14,29]. In contrast, their progeny—second generation homozygotes—develop slowly at 15–20$^\circ$C, to form infertile adults that are far more stress resistant and at least four-fold longer lived than other IIS mutants [30]. Maternal protection (oocyte carryover of \textit{age-1} mRNA, AGE-1 kinase or its PIP3 product, synthesized by the heterozygous parent) is thought to blunt both the stress-resistance and longevity traits to approximately those of a weaker \textit{age-1} or \textit{daf-2} allele [30]. We here show that total kinase activity is also attenuated more severely in \textit{age-1(mg44)} adults at the \textit{F2} than the
Consequences of altered gene expression for IIS and other pathways

Transcriptional effects within the IIS pathway seem fully consistent with impaired insulinlike signaling, which might be expected to further augment survival through the same mechanisms employed by weaker IIS mutations. Moreover, repression of class-II and class-III PI3K catalytic-subunit genes (Table 1) would impede formation of P1(3,3)P and P(3,4,5)P2, suppressing alternative routes to P(3,4,5)P2. Increased expression of aak-2 contributes to activation of DAF-16/FOXO, further opposing IIS (which inhibits this transcription factor) and increasing life span [43,44].

In addition to effects on IIS genes, however, age-1(mg44)-F2 adults also show striking transcriptional attenuation of several other signal transduction pathways that interact with IIS and with one another. TGF-β endocrine/paracrine signaling is active in development, and modulates several other signaling pathways including p38/MAPK and ERK/MAPK [46]. Bothdaf-1 anddaf-4, encoding type-1 and -II TGF-β receptors, respectively, are downregulated 4- to 5-fold in age-1(mg44)-F2 adults. Expression is also reduced 3-fold fordaf-3, encoding a co-SMAD transcription factor deployed by several pathways including TGF-β. Perhaps in partial compensation for this signaling downregulation, thedaf-7 gene encoding a TGF-β-family ligand/agonist is 5-fold upregulated. Silencing of TGF-β signaling by RNAi directed atdaf-3 ordaf-4, improves survival in the presence of hydrogen peroxide, consistent with a prior observation thatdaf-1, -4 and -7 mutants are long-lived [46].

AMPK/TOR signaling has been implicated in innate immunity and stress responses. Although it remains controversial whether the primary response is to the microbe or to the stress it causes [47], both could be secondary to its role in nutrient sensing [37,43]. AAK-1 and AAK-2 are regulated by the PAR-4 transcription factor [37,58,59], and aak-1 knockdown by RNAi confers resistance to oxidative stress (Figure 5C). Inhibition of theC. elegans TOR pathway confers stress resistance and extends life span [60,61].

ERK/MAPK signal-transduction is essential for many developmental processes; because the constituent genes are also expressed in adult nematode tissues, they are presumed to have post-developmental functions not yet defined [62–64]. All six genes tested in this pathway are markedly downregulated, by 3- to 6-fold (Table 1), and RNAi inhibition oflet-60 (encoding a RAS membrane co-receptor that initiates ERK/MAPK signaling) significantly improves survival of oxidative stress (Figure 5C).

Rationale, anomalies, and resolutions

The most dramatic effects of gene mutations on life span have involved hypomorphic (loss-of-function) mutations, and the genes affected have been termed aging “master genes”. The genes encoding IIS components provide the best-studied example. IIS, in common with many “master genes” and essentially all signaling pathways, regulates numerous other genes. In the case of IIS, a number of these are modulated in ways that are protective, or otherwise conducive to long life, such as upregulation of GSTs and other detoxification genes, which are among the “foot soldiers” of longevity assurance [65,66]. However, we should not expect all such downstream consequences to confer uniformly pro-longevity effects; each gene is likely to serve several “masters”, and its level of expression will depend on the genetic, environmental, and signaling context. In keeping with this perspective, the downstream manifestations of longevity assurance genes are far less conserved, both in evolution and between distinct physiological states of a given species, than are the over-arching pathways and the functions they serve [67].

Improved stress resistance and survival of age-1(mg44)-F2 worms, apparently arising from transcriptional attenuation of signaling pathways presumed to be protective, poses an intriguing paradox. These pathways, activated by nutrient deficiency, pathogens, or growth factors, have been reported to cross-talk with IIS at diverse levels [19,43–47,68,69]. This suggests a complex fabric of signaling interactions, for which the impact of silencing multiple components cannot be predicted. Moreover, signaling that promotes survival in a variable or hostile setting may entail energy costs and harmful side-effects that would be unwarranted in a constant, pathogen-free environment with abundant food. An organism that avoids the deleterious aspects of these surveillance systems may thus reap survival benefits under benign conditions.

In several instances, the expression changes seen in strong age-1 mutants appear to oppose their longevity or stress-resistance, based on the effects of down- or upregulation previously reported for the same genes. For example, age-1(mg44) F2 adults downregulatedaf-18, which encodes the PIP3-phosphatase, PTEN. This would be expected to elevate the steady-state level of PIP3, thereby enhancing IIS and reducing longevity of normal worms. However, in the absence of AGE-1/PI3KCIIa kinase activity, there may be little or no PIP3 substrate on which PTEN could act. A second example is downregulation in age-1(mg44) F2 adults ofskn-1, encoding a transcription factor responsive to oxidative damage and regulated via IIS [70–72]. Reduced expression ofskn-1 seems at odds with increased oxidative-stress resistance and longevity; however, these very long-lived worms may generate lower levels of reactive oxygen species, thereby reducingskn-1 induction. RNAi tovps-34 (encoding a class-III PI3KCIIIb required for vesicular trafficking and autophagy [73]) was recently shown to block life extension ofeat-2(ad1116) anddaf-2(mu150) mutants, although not of wild-type worms [58]. Autophagy is induced by TOR deficiency [58], and several TOR signaling components are downregulated in F2 worms (Table 1). Considering this, autophagy should be at least moderately induced in those worms, and its absence would not account for low expression ofvps-34. These results argue against any direct role ofvps-34 attenuation in the exceptional longevity of age-1(mg44) F2 worms. The possibility remains, however, thatvps-34 downregulation could reinforce PIP3-depleting effects of a strong age-1 mutation. Downregulation in age-1(mg44) worms, of transcripts forlet-60/RAS and five other members of the ERK-MAPK cascade, might be expected to oppose additional life extension beyond that typical of IIS mutants, because alet-60 gain-of-function mutation enhancesdaf-2 life extension [74]. RNAi targetingsmk-1, encoding a transcriptional coactivator shared by DAF-16 and PHA-4 [75], reduces stress-resistance and lifespan ofdaf-2(e1370) worms [76], whereas the effect on wild-type worms is controversial [34,76]. Althoughsmk-1 knockdown impairssod-3 expression indaf-2 worms [34], we found 9-fold elevation of sod-3 transcripts in the face of a72% drop insmk-1 expression inage-1(mg44) (Table 1). Finally,sir-2.1 overexpression was reported to extend lifespan, and knock-down to shorten it [77,78], whereas we found almost 4-fold downregulation ofsir-2.1 in age-1(mg44)-F2 adults.

Although contradictory in the context of extreme stress resistance and longevity, all six of these “exceptions” are also
mirrored, in most cases to a lesser degree, in dauer larvae (Table 1), a robust state of developmental arrest that can endure for months without reducing adult life span [1,79,80]. This raises the possibility that for these genes, the effects of downregulation are context-dependent, and may be beneficial in worms that are already highly protected from stress and aging. Alternatively, these expression changes may follow from regulatory mechanisms shared by age-1(mg44) adults and N2 dauer, and yet work in opposition to their robustness. This is plausible in the case of a severe loss-of-function mutant, effects of which are not orchestrated, but is difficult to reconcile with a highly-evolved alternative developmental state such as the dauer larva. Perhaps, rather than a single coherent program, the patterns we observe reflect aberrant triggering in the adult of one or more regulatory mechanisms that are normally utilized in developmental or metabolic regulation. This particular combination of mechanisms could be the serendipitous result of a profound alteration in PI3K levels which in turn impacts multiple pathways.

**PI3Kcs-mutant effects are largely, but not entirely, mediated by DAF-16/FOXO**

The expression profile of age-1(mg44) worms depends largely on DAF-16/FOXO, consistent with prior evidence that C. elegans IIS operates mainly through this transcription factor, impacting several hundred target genes [2,21,22,29,52,81]. Although DAF-16/FOXO has been regarded largely as a transcriptional activator [21,82], it also affects negative regulation of many genes [24]. In our selected panel of genes, two-thirds (22/33) of the DAF-16-mediated effects of age-1(mg44) mutation involve reduced transcript levels, indicating that silencing prevails for gene transcripts that encode kinases and other mediators of intracellular signaling. Twenty-eight genes (of the 33 for which transcripts appear to be primarily regulated via DAF-16/FOXO) were mapped for DAF-16 binding sites within 5 kb upstream of the initiation codon (Table 1). Of these, 21 (75%) have exact matches to one or both of the two known consensus sites, GTAAA(C/A)AA and CTTATCA. Genes lacking such sites may be indirect targets of DAF-16/FOXO, but considering that those motifs occur at almost the same frequency in the genome at large [81], as in DNA immunopre-cipitated with antibody to DAF-16/FOXO [52], it is possible that precise motif matches are neither necessary nor sufficient for DAF-16 binding. In other words, near-match sequences might be able to bind DAF-16, while even perfect-match motifs may require additional features in nearby DNA.

**Implication of a hybrid feedback loop with kinase and transcriptional components**

It is surprising that hyperactivation of DAF-16/FOXO in age-1(mg44) F2 adults silences essentially the entire IIS pathway. This implies a positive feedback loop, in which DAF-16/FOXO imposes transcriptional silencing on the very kinases that would inhibit its own nuclear localization and hence access to target genes (Figure 6). We propose that second-generation age-1(mg44) homozygotes are trapped in a nonadaptive state, incapable of responding to diverse environmental and internal signals. This apparent paradox, that failure of adaptive mechanisms greatly extends lifespan, is easily explained because those mechanisms maximize Darwinian fitness — transmission of genetic alleles to ensuing generations — rather than individual survival [83,84].

When IIS kinase signaling predominates (the reproductive state), it suppresses DAF-16/FOXO activity. Activation of PI3K favors PI3P production and AKT activation, both of which promote cell proliferation [18,31,85]. However, IIS can switch to a second, functionally distinct, state: when kinase signaling is weak, DAF-16/FOXO becomes activated. As we have demonstrated, active DAF-16/FOXO transcriptionally silences its own upstream regulatory kinases, which otherwise would have impeded DAF-16/FOXO action by preventing its nuclear localization. Therefore, the low-signaling, longevity state of IIS is self-sustaining. Biologically, this state promotes dauer formation during development, or life-extension and delayed reproduction in the adult (reviewed in [4]). Signals that inhibit IIS kinases or augment DAF-16/FOXO action, if sufficient, trigger a switch from reproductive to longevity state in which DAF-16/FOXO promotes somatic protective mechanisms (Figure 6). However, exiting the stable longevity mode requires a shift in the balance of inputs that govern the positive feedback loop. Such inputs may include insulin-like peptide agonists and antagonists, hormones, pheromones, tran-

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**Figure 6. Model of the IIS “molecular switch.”** Three proposed system states are depicted, reflecting the balance between kinase signaling and transcriptional feedback suppression: reproductive mode, in which kinase signaling predominates, activated via the insulin/IGF-1 receptor (DAF-2, here labeled Ins-R); longevity mode, wherein FOXO (DAF-16) prevails and suppresses kinase transcription; and hyperlongevity mode—in which switching between the first two modes is blocked, thus strongly favoring survival but with no possibility of resuming reproduction. State transitions are normally triggered by signaling modulators (e.g., insulin-like peptides [12] or SIR-2/14-3-3 complex [77]), to which age-1(mg44)-F2 mutants (“hyperlongevity” state) cannot respond.

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scripctional co-activators and co-repressors of DAF-16/FOXO such as SIR-2 and 14-3-3 proteins, and nutrient- and stress-sensors signaled though other kinase pathways (e.g., MAPK, JNK and AMPK) that cross-talk with IIS. Combined, these two normal states of the IIS pathway (reproductive and longevity) constitute a “flip-flop” circuit with opposing kinase-cascade and transcriptional signals (Figure 6).

The concept of a “genetic switch” for dauer formation is not new [1,86,87], and has even been demonstrated to constitute a bistable feedback loop [21,87]. Nevertheless, a dual-level (kinase/transcriptional) feedback mechanism had not previously been proposed or described. Any such “flip-flop” circuitry allows the organism a simple binary choice in response to its environment: early reproduction under benign conditions, or postponed reproduction and extended survival in harsher conditions. Mutational disruption of IIS forces dauer formation, irrespective of environment, although larvae with temperature-sensitive mutations can mature at lower temperatures into long-lived adults. Recovery from the dauer state requires that pro-reproductive-state kinases retain partial function, so that favorable signals (restoration of food, absence of stress and crowding) can reset the switch to the reproductive mode; this requirement is demonstrated by the impaired post-dauer recovery of IIS-defective mutants [86]. Nonsense mutations truncating AGE-1 produce an extreme phenotype that forfeits this option, while acquiring a distinctive transcriptional profile and greatly enhanced survival. Details of the mechanism or mechanisms, by which elimination of PI3K activity blocks exit from the longevity mode and promotes extreme longevity, remain to be elaborated. Features described in this report, which may contribute, include transcriptional silencing of upstream and collateral signaling components, and accompanying loss of multiple kinase activities. Infertile mutants may thus reveal new and collateral signaling components, and accompanying loss of strategies to extend life well beyond the limits imposed by natural selection, which of course requires reproduction. In view of the striking evolutionary conservation of the IIS pathway, and the emerging parallels between inter-pathway cross-talk in nematodes and mammals [16,19,47,60,69], the mechanistic insights afforded by very long-lived worms are likely to also apply to insulin and IGF-1 pathways of mammals.

Materials and Methods

Strains

Nematode strains, supplied by the Caenorhabditis Genetics Center (CGC, Minneapolis), or derived in our laboratory from CGC strains, were maintained at 20°C on 0.6% peptone NGM-agar plates seeded with E. coli strain OP50, as described [88–90].

Stress resistance

Assays of survival in the presence of 5-mM hydrogen peroxide or 10-mM 4-hydroxynonenal were modified from Ayyadewara et al. [91]. Wild-type (N2DRM) worms were assayed at day 3–4 of adulthood (~6 d post-hatch). For RNAi experiments, day-1 adults were washed in S-buffer [92] and transferred to nutrient-agar plates seeded with dsRNA-expressing E. coli [56]. After 3 days at 20°C, 20 worms from each RNAi treatment were transferred to 24-well plates containing 300 μl of S Buffer plus 5 μg/ml cholesterol, supplemented, as indicated, either with 5-mM H2O2 (freshly diluted from 30% H2O2, Sigma) or with 10-mM 4-HNE (freshly obtained by acid hydrolysis of 4-HNE dimethylacetal which was synthesized according to [93]. Survival was scored as described [30,89].

In vitro phosphorylation assay

Worms grown at 20°C were quickly frozen in liquid nitrogen to preserve endogenous kinase activity. Worms suspended in 50-mM Tris pH 7.5, 80-mM β-mercaptoethanol, 2-mM EDTA, 1-mM PMSF, and Protease Inhibitor Cocktail I (CalBiochem), were ground at −78°C and sonicated (VIRTIS Vironsic 475, setting 2.5, 0°C) in six 10-s bursts interspersed with 2-min cooling periods. Kinase activity toward endogenous substrates was assessed in cleared supernatants after centrifugation (10 min, 11,000 g), representing 20 μg protein in 100 μl of buffer containing 50-mM Tris pH 7.5, 12.5-mM MgCl2 and (for endogenous substrates) 8–10 μCi γ-32P-ATP (NEN). After 1 min at 30°C, quenched samples were electrophoresed on 10% SDS-polyacrylamide gels (Invitrogen), which were stained with SYPRO Ruby (Invitrogen), and dried under vacuum. 32P β-emissions of bands migrating slower than a 25-kDa protein marker (Invitrogen), were imaged and quantified per lane after 6-h phosphor-screen exposure (Storm, Molecular Dynamics). Peptide arrays were incubated as above, 60 min at 30°C, but with addition of phosphatase inhibitors and 1-mM cold ATP rather than 32P-ATP. Arrays were then stained with Pro-Q Diamond (Invitrogen), and phosphorylation was quantified by fluorescence imaging (excitation/emission at 530/580 nm) with a ScanArray 5000 (GSI Lumonics).

In vivo phosphoprotein detection and quantitation

Total protein (20 μg), extracted from each strain as above, was loaded onto NuPAGE 4–12% gradient gels (Invitrogen) and electrophoresed 1 hour at 200 V. Phosphoproteins were quantified by Pro-Q Diamond (Invitrogen) fluorescence, which depends linearly on protein concentration (>1000-fold range). Protein load was assessed by Coomassie Blue (BioRad) staining. Phosphorylated (23.6, 45.0 kDa) and unphosphorylated (14.4, 18.0, 62.6, 116.2 kDa) protein standards (BioRad) furnished positive and negative controls.

Transcript quantitation by RT–PCR

Expression of selected genes was assessed by real-time polymerase chain reaction after an initial round of reverse transcription. Total RNA was purified from each strain (RNeasy, Qiagen), and cDNAs reverse-transcribed (SuperScript III, Invitrogen), followed by RT–PCR (Opticon2, MJ Research, using SYBR Green, Roche).

Supporting Information

Figure S1 In vitro kinase activity for endogenous substrates is reduced in age-1(mg44) homozygotes, but not in dauer larvae. Kinase activity was assessed for N2DRM day-6 adults and eggs they produce, eggs laid by age-1(mg44) F1 adults, F1 adults at days 2.5 and 6.5 of adulthood, age-1(mg44) F2 day-1 adults, daf-16(mu86); age-1(mg44) adults, and N2DRM dauer larvae. Kinase activity of sonicated lysates was assessed as described in the legend to Figure 1. Only a single biological sample was assessed for each group (hence no error bars are shown); technical replicates agreed within ±20%, and replicate experiments were consistent with results shown. Found at: doi:10.1371/journal.pgen.1000452.s001 (1.42 MB TIF)

Figure S2 Phosphorylation in vitro of peptide arrays. Fluorescence images are displayed of peptide arrays (JPT Peptide Technologies GmbH, Berlin) shown printed in duplicate blocks, stained with Pro-Q Diamond (Invitrogen) after 1-h incubation with worm homogenates containing phosphatase and protease
inhibitors and 1-mM ATP. Equal protein concentrations from each strain (N2DRM, age-1(mg44), and daf-2(e1693(mg231); age-1(mg44)) were incubated on slides, 60 min at 30°C. Note that “reversion” by daf-16(mu86) enhances some kinases not evident even in the wild-type (N2DRM), sample.

Found at: doi:10.1371/journal.pgen.1000452.s002 (0.77 MB TIF)

**Figure S3** Adult age-1(mg44) F2 homozygotes are deficient in PI3Kcs protein. Western blots are shown, after denaturing (A) or native (B) polyacrylamide gel electrophoresis. Electrochemiluminescence (GE-Amersham “ECL-plus”) detected secondary antibody (goat anti-rabbit IgG coupled to horseradish peroxidase) to rabbit monoclonal antibodies binding the C-terminal region of C. elegans phosphatidylinositol 3-kinase p110 catalytic subunit, or to cytoplasmic β-actin as a loading control (Santa Cruz Biotech., Santa Cruz CA). Each lane contains 10 μg of total protein.

Found at: doi:10.1371/journal.pgen.1000452.s003 (0.25 MB TIF)

**Table S1** Peptide phosphorylation by *C. elegans* kinases in vitro. Mean±SD is given for ProQ Diamond fluorescence (in arbitrary units) of synthetic peptides spotted in 2–4 arrays, after subtraction of mean background measured on negative-control spots. The kinases that phosphorylate these peptides are not known; those listed in column 2 may phosphorylate the residues underlined, based on consensus sites for the corresponding mammalian kinase.

**References**

1. Riddle DL, Swanson MM, Albert PS (1991) Interacting genes in nematode dauer larva formation. Nature 290: 668–671.
2. Larsen PL, Albert PS, Riddle DL (1995) Genes that regulate both development and longevity in *Caenorhabditis elegans*. Genetics 139: 1567–1583.
3. Dorman JB, Albinier B, Shroyer T, Kenyon C (1995) The age-1 and daf-2 genes function in a common pathway to control the lifespan of *Caenorhabditis elegans*. Genetics 141: 1399–1406.
4. Kenyon C (2005) The plasticity of aging: insights from long-lived mutants. Cell 120: 499–506.
5. Friedman DB, Johnson TE (1988) A mutation in the age-1 gene in *Caenorhabditis elegans* lengthens life and reduces hirymphodrude fertility. Genetics 118: 75–86.
6. Kenyon C, Chang J, Gensch E, Rudher N, Tatian R (1993) A *C. elegans* mutant that lives twice as long as wild type. Nature 366: 461–464.
7. Li K, Doernan JR, Rodan A, Kenyon C (1997) daf-16: An IFN-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. Science 278: 1319–1322.
8. Tissenbaum HA, Ruvkun G (1998) An insulin-like signaling pathway affects both longevity and reproduction in *Caenorhabditis elegans*. Genetics 148: 703–717.
9. Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, et al. (2001) Extension of Life-Span by Loss of CHICO, a Drosophila Insulin Receptor Substrate Protein. Science 292: 104–106.
10. Tatar M, Koppelman A, Epstein T, Dv MP, Yin CM, et al. (2001) A Mutant Drosophila Insulin Receptor Homolog That Extends Life-Span and Impairs Neuroendocrine Function. Science 292: 107–110.
11. Barkal AJ, Wright JC, Mattison JA, Ingram DK, Miller RA, et al. (2001) Extending the lifespan of long-lived mice. Nature 414: 412.
12. Pierce SB, Costa M, Wisotzkey R, Devadhar S, Hamburger SA, et al. (2001) Regulation of DAF-12 reporter signaling by human insulin and irx-1, a member of the unusually large and diverse *C. elegans* insulin gene family. Genes Dev 15: 672–686.
13. Paradis S, Ailion M, Toker A, Thomas JH, Ruvkun G (1999) A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in *Caenorhabditis elegans*. Genes Dev 13: 1438–1452.
14. Paradis S, Ruvkun G (1998) *Caenorhabditis elegans* Akt/PI3K transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes Dev 12: 2488–2498.
15. Hemmings BA (1997) PtdIns(3,4,5)P3 gets its message across. Science 277: 534.
16. Ailion M, Toker A, Thomas JH, Ruvkun G (1999) A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in *Caenorhabditis elegans*. Genes Dev 13: 1438–1452.
17. Paradis S, Ruvkun G (1998) *Caenorhabditis elegans* Akt/PI3K transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes Dev 12: 2488–2498.
18. Hemmings BA (1997) PtdIns(3,4,5)P3 gets its message across. Science 277: 534.
19. Singh SS, Chauhan A, Brockerhoff H, Chauhan VP (1993) Activation of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. Biochem Biophys Res Commun 195: 104–112.
20. Stokoe D, Stephens LR, Copeland T, Galloway PRJ, Reves CB, et al. (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of Protein Kinase B. Science 277: 567–570.
21. Shultzans V, Wu M, Burstein DE (2008) Current overview of the role of Akt in cancer studies via applied immunohistochemistry. Ann Diagn Pathol 12: 153–160.
22. Gami MS, Iser VB, Hanselman KB, Wolkow CA (2006) Activated AKT/PI3K signaling in *C. elegans* uncouples temporally distinct outputs of DAF-2/insulin-like signaling. BMC Dev Biol 6: 45.
formation in an insulin receptor-like signaling pathway. Proc Natl Acad Sci U S A 96: 7427–7432.

39. Kawano T, To Y, Ishiguro M, Takaua K, Nakajima T, et al. (2000) Molecular cloning and characterization of a new insulin/IGF-like peptide of the nematode Caenorhabditis elegans. Biochem Biophys Res Commun 273: 431–436.

40. Chakravarti K, Casuto H, Reshof L, Hanson RW (2005) Factors that control the tissue-specific transcription of the gene for phosphophenylpyruvate carbonic anhydrase-C. Crit Rev Biochem Mol Biol 40: 129–154.

41. Wolkow CA, Munoz MJ, Riddle DL, Rudkin G (2002) Insulin receptor substrate and p53 orthogonal adaptor proteins function in the Caenorhabditis elegans daf-2/insulin-like signaling pathway. J Biol Chem 277: 49591–49597.

42. Denley A, Carroll JM, Brierley GV, Cosgrove I, Wallace J, et al. (2007) Differential activation of insulin receptor substrates 1 and 2 by insulin-like growth factor-activated insulin receptors. Mol Cell Biol 27: 3569–577.

43. Greer EL, Dowlatshahi D, Banko MR, Villen J, Hoang K, et al. (2007) An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. Curr Biol 17: 1646–1656.

44. Apfeld J, O'Connor G, McDonagh T, DiStefano PS, Curtis R (2004) The AMP-activated kinase AAK-2 links energy levels and insulin-like signals to lifespan in C. elegans. Genes Dev 18: 3004–3009.

45. Curtis R, O'Connor G, DiStefano PS (2006) Aging networks in Caenorhabditis elegans: AMP-activated protein kinase (aak-2) links multiple aging and metabolism pathways. Aging Cell 5: 119–126.

46. Shaw WM, Luo S, Landis J, Shafafy J, Murphy CT (2007) The C elegans TGF-beta Dauer pathway regulates longevity via insulin signaling. Curr Biol 17: 1635–1645.

47. Troemel ER, Chu SW, Reinke V, Lee SS, Ausubel FM, et al. (2006) p38 MAPK regulates expression of immune response genes and contributes to longevity in C. elegans. Proc Natl Acad Sci U S A 103: 11833–11838. doi:10.1073/pnas.0602172103.

48. Oh SW, Mukhopadhyay A, Svrzikapa N, Jiang F, Davis RJ, et al. (2005) JNK-mediated activation of insulin receptor substrates 1 and 2 by insulin-like growth factor-activated insulin receptors. Mol Cell Biol 25: 3569–3577.

49. Helfand SL, Dowlatshahi D, Banko MR, Villen J, Hoang K, et al. (2007) An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. Curr Biol 17: 1646–1656.

50. McElwee JJ, Schuster E, Blanc E, Thomas JH, Gems D (2004) Shared regulatory interactions among conserved longevity assurance mechanisms in Caenorhabditis elegans. Proc Natl Acad Sci U S A 101: 1203–1208. doi:10.1073/pnas.0306985101.

51. Lee H, Cho JS, Lambacher N, Lee J, Lee SJ, et al. (2008) The daf-16/mth-1 gene regulates expression of immune response genes and contributes to longevity in Caenorhabditis elegans. J Biol Chem 283: 14988–14993.

52. Kim SK (2007) Common aging pathways in worms, flies, mice and humans. Aging Cell 6: 95–110.

53. Hirsh D (1976) Non-aging developmental variant of Caenorhabditis elegans. 260: 525–525, Nature 260: 525–525.

54. Sironi L, Tissenbaum HA (2006) Overlapping and distinct functions for a Caenorhabditis elegans SIR-2 and DAF-16/FOXO pathway in aging. Mech Ageing Dev 127: 41–56.

55. Zou J, Zhang J, Wang L, Chen X, Liu CL (1994) Adiponectin: a novel adipose-specific protein produced in response to insulin stimulation and converse secreted by adipocytes. J Biol Chem 269: 5325–5329.

56. Kamath RS, Ahringer J (2003) Genome-wide RNAi profiling of early embryogenesis in Caenorhabditis elegans. Genome Res 13: 2782–2792.

57. Kim SK (2007) Common aging pathways in worms, flies, mice and humans. J Biol Chem 283: 14988–14993.

58. Ayyadevara S, Ayyadevera R, Hou S, Thaden JJ, Shmookler Reis RJ (2001) DAF-16 regulates expression of immune response genes and contributes to longevity in C. elegans. Proc Natl Acad Sci U S A 101: 1203–1208. doi:10.1073/pnas.0306985101.

59. Lee MH, Hook B, Pan G, Kershner AM, Merritt C, et al. (2007) Conserved autophagy in the extension of lifespan by dietary restriction in Caenorhabditis elegans. PLoS Genet 3: e12. doi:10.1371/journal.pgen.0040012.

60. Lee H, Cho JS, Lambacher N, Lee J, Lee SJ, et al. (2008) The daf-16/mth-1 gene regulates expression of immune response genes and contributes to longevity in Caenorhabditis elegans. J Biol Chem 283: 14988–14993.

61. Lee H, Cho JS, Lambacher N, Lee J, Lee SJ, et al. (2008) The daf-16/mth-1 gene regulates expression of immune response genes and contributes to longevity in Caenorhabditis elegans. J Biol Chem 283: 14988–14993.

62. Kim SK (2007) Common aging pathways in worms, flies, mice and humans. J Biol Chem 283: 14988–14993.

63. Sundaram MV (2006) RTK/Ras/MAPK signaling. WormBook 1–19.

64. Lamitina ST, Strange K (2005) Transcriptional targets of DAF-16 insulin like growth factor-1 signaling in Caenorhabditis elegans. PLoS Biol 3: e1000002. doi:10.1371/journal.pbio.0000002.

65. Castillo SS, Brognard J, Petukhov PA, Zhang C, Tsurutani J, et al. (2004) Mutation of protein kinase C in regulating nuclear translocation of DAF-16 and extending life span. Cell 121: 1165–1177.

66. Ebert RH, Cherkasova VA, Dennis RA, Wu JH, Ruggles S, et al. (1993) Mutagenic effects of 2,6-dinitrotoluene on the tissues of adult Caenorhabditis elegans. Proc Natl Acad Sci U S A 90: 4333–4338.

67. Singh SP, Chen T, Chen L, Mei N, McLain E, et al. (2005) Mutagenic effects of 2,6-dinitrotoluene on the tissues of adult Caenorhabditis elegans. Proc Natl Acad Sci U S A 90: 4333–4338.

68. Ayyadevara S, Ayyadevera R, Hou S, Thaden JJ, Shmookler Reis RJ (2001) DAF-16 regulates expression of immune response genes and contributes to longevity in C. elegans. Proc Natl Acad Sci U S A 101: 1203–1208. doi:10.1073/pnas.0306985101.

69. Nath J, Hopper N, Gens D (2005) LET-60 RMS modulates effects of insulin/IGF-1 signaling on development and aging in Caenorhabditis elegans. Aging Cell 4: 235–245.

70. Panzovski SH, Wolff S, Aquilanis H, Durieux J, Dillin A (2007) PHA-4/FOXO mediates diet-restriction-induced longevity of C. elegans. Nature 447: 550–553.

71. Tullet JM, Hertseck M, An JH, Baker J, Hwang JY, et al. (2008) Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. Cell 132: 1025–1038.

72. Belin NA, Guarente L (2007) Two neurons mediate diet-restriction-induced longevity in C. elegans. Nature 447: 545–549.

73. Lomeni A, Kuchi N, Greco MM, Yudkoff M, Smith E, et al. (2007) A groundbreaking study on the effects of dietary restriction on aging in C. elegans. PLoS One 2: e1137. doi:10.1371/journal.pone.0000113.

74. Nanji M, Hopper N, Gens D (2005) Testing evolutionary change using the hypersensitive rrf-3 strain of Caenorhabditis elegans. Categorical hypotheses in the theories of aging. Ann N Y Acad Sci 908: 319–320.

75. Ayyadevera S, Vertino A, Galecki A, Thaden JJ, Shmookler Reis RJ (2001) DAF-16 regulates expression of immune response genes and contributes to longevity in C. elegans. Proc Natl Acad Sci U S A 90: 4333–4338.