A systematic RNAi screen for longevity genes in *C. elegans*

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We report here the first genome-wide functional genomic screen for longevity genes. We systematically surveyed *Caenorhabditis elegans* genes using large-scale RNA interference (RNAi), and found that RNAi inactivation of 89 genes extend *C. elegans* lifespan. Components of the *daf-2*/insulin-like signaling pathway are recovered, as well as genes that regulate metabolism, signal transduction, protein turnover, and gene expression. Many of these candidate longevity genes are conserved across animal phylogeny. Genetic interaction analyses with the new longevity genes indicate that some act upstream of the *daf-16*/FOXO transcription factor or the *sir2.1* protein deacetylase, and others function independently of *daf-16*/FOXO and *sir2.1*, and might define new pathways to regulate lifespan.

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Diverse genetic and environmental factors regulate longevity in a wide range of organisms (Finch 1990), but features of lifespan control that might be universal to animals have begun to emerge (Hekimi and Guarente 2003; Tatar et al. 2003). In *Caenorhabditis elegans*, modulations of particular gene activities cause a profound increase in lifespan (Guarente and Kenyon 2000). Most notably, reduced activity of the *C. elegans daf-2*/insulin-like signaling pathway causes a more than twofold lifespan increase (Kenyon et al. 1993; Morris et al. 1996; Kimura et al. 1997; Paradis and Ruvkun 1998; Paradis et al. 1999). Signaling from the DAF-2/insulin-like receptor antagonizes the forkhead (FOXO) transcription factor *daf-16*, the major effector of *daf-2*/insulin-like regulation of *C. elegans* lifespan (Lin et al. 1997; Ogg et al. 1997). Similar modulations of insulin-like signaling pathways in fruit fly and mouse also modify lifespan (Clancy et al. 2001; Tatar et al. 2001; Bluher et al. 2003; Holzenberger et al. 2003), indicating that this pathway is a universal longevity regulator. There are >30 insulin genes in *C. elegans* that might mediate input to the *daf-2* pathway through environmental cues, such as nutritional status or growth conditions (Pierce et al. 2001). Consistent with this model, mutations abrogating sensory neurons also regulate *C. elegans* lifespan in a *daf-16*-dependent manner (Apfeld and Kenyon 1999).

The Sir2 histone deacetylase is another important longevity determinant that regulates lifespan in diverse species (Kaeberlein et al. 1999). In yeast, Sir2 facilitates heterochromatin formation, and by reducing recombination at the rDNA locus, Sir2 helps to maintain genomic stability and extends lifespan (Imai et al. 2000). Similar to yeast, overexpression of the *C. elegans SIR2* homolog *sir-2.1* also extends lifespan (Tissenbaum and Guarente 2001), although the molecular mechanism whereby *sir-2.1* enhances longevity in nematode is less clear. Genetic studies indicate that *sir-2.1* works upstream of *daf-16* to regulate *C. elegans* lifespan (Tissenbaum and Guarente 2001). Consistent with this genetic placement, in mammals, the Sir2 homolog SIRT1 deacetylates FOXOs, the mammalian DAF-16 homologs, and directly modulates FOXOs activity (Brunet et al. 2004; Giannakou and Partridge 2004; Motta et al. 2004; van der Horst et al. 2004).

Interestingly, mutations inhibiting *C. elegans* germline proliferation also extend lifespan (Hsin and Kenyon 1999; Arantes-Oliveira et al. 2002). The germline produces a *daf-16*-dependent signal to regulate lifespan, but acts independently of the upstream *daf-2* gene (Lin et al. 2001). Thus, *daf-16* might act as a master regulator of lifespan, capable of integrating several different longevity signals.

A handful of genes have been found to regulate *C. elegans* lifespan through pathways independent of *daf-16*. The feeding-defective *eat* mutants live slightly longer in

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a *daf-16*-independent manner. The *eat* mutations are suggested to extend lifespan through a mechanism resembling caloric restriction (Lakowski and Hekimi 1998). Mutations in *clk-1*, a gene required for coenzyme Q synthesis, also extends lifespan independently of *daf-16*, and slow the rate of many physiological processes (Lakowski and Hekimi 1996). Similarly, reduced function of the mitochondrial oxidative phosphorylation machinery extends *C. elegans* lifespan independent of *daf-16* (Feng et al. 2001; Dillin et al. 2002; Lee et al. 2003b). A small-scale RNA interference (RNAi) screen for longevity regulators revealed that inactivation of particular mitochondrial electron transport chain (ETC) components increases *C. elegans* lifespan (Lee et al. 2003b).

Despite the identification of many genes that can regulate lifespan, a comprehensive survey of all of the gene classes capable of limiting the longevity of an animal has not been reported. We report here a genome-wide RNAi lifespan screen and the identification of a large number of new longevity genes, many of which are highly conserved in other species. This screen reveals 89 gene inactivations that can extend *C. elegans* lifespan from 5% to 70%. Our screen represents the first genome-wide survey of gene inactivations that can prolong *C. elegans* lifespan and provides a global view of the gene classes and physiological processes that can regulate the longevity of an animal. The large number of new longevity regulators reported here provides an important entry point for further characterizations that will likely reveal important insights into the molecular control of organismal longevity.

Results and Discussion

Genome-wide RNAi screen

Using an RNAi bacterial library targeting >80% of the ~19,000 *C. elegans* open reading frames (Fraser et al. 2000; Kamath et al. 2003) and a high-throughput lifespan assay, we screened for RNAi inactivations that extend the lifespan of wild-type N2 worms. The RNAi clones that were scored positive in the first round of primary screen were subjected to two more rounds of high-throughput lifespan assay. Of the 16,475 RNAi clones tested, ~600 RNAi clones induced lifespan extension above a rather modest threshold in high-throughput screening. Of these ~600 RNAi clones, we performed lifespan assays with many more timepoints using *rrf-3(pk1426)*, a mutant with enhanced susceptibility to feeding RNAi (Simmer et al. 2002) and a normal lifespan (Lee et al. 2003a; Murphy et al. 2003). After three rounds of retesting, we identified 90 RNAi clones that significantly extended *C. elegans* lifespan (P-value < 0.05) in at least two of the lifespan experiments (Table 1; Supplementary Table 1). The plasmid construct for each of the final 90 RNAi clones was isolated and sequenced to verify its corresponding target gene (Table 1). The 90 RNAi constructs target 89 distinct genes, because RNAi constructs C01F6.7 and F23B2.1 both target the C01F6.6 gene. Significantly, most of the candidate longevity genes are conserved in evolutionarily diverse species. In fact, based on Ensembl annotations (http://www.ensembl.org), 32 of the candidate genes have a clear orthologous partner in *Drosophila*, mouse, and/or human. Even for the genes that are not annotated to have a clear ortholog, many of them display significant sequence homology to genes in diverse species. This finding suggests that characterization of the candidate longevity genes identified in our screen will likely reveal mechanisms that influence longevity in many different organisms.

The efficacy of our RNAi screen is validated by the recovery of genes and gene classes previously characterized to regulate *C. elegans* lifespan. We found that RNAi of *age-1* as well as *akt-1*, both of which are components of the *daf-2*/insulin-like signaling pathway, caused robust lifespan extension (Table 2; Supplementary Table 1). *age-1* mutations are well-established to extend lifespan (Morris et al. 1996). Interestingly, although *akt-1* is a well-characterized component of the *daf-2* signaling pathway, an *akt-1* deletion mutant was previously reported to exhibit normal lifespan (Hertweck et al. 2004). The lifespan extension phenotype caused by *akt-1* RNAi (Table 2) might only be apparent when *akt-1* activity is reduced by RNAi knockdown, and not when *akt-1* function is completely lost, as in the case of a deletion mutant. An alternative hypothesis that nonspecific knockdown of additional gene(s) by the *akt-1* RNAi construct is also possible, though Blastn analysis reveals additional genes with only 17 bp of sequence identity to *akt-1*.

We also found that RNAi of several subunits of the mitochondrial ETC significantly extended *C. elegans* lifespan. This is consistent with previous genetics and RNAi studies indicating that reduced mitochondrial electron transport can lead to lifespan increase in *C. elegans* (Feng et al. 2001; Dillin et al. 2002; Lee et al. 2003b).

Longevity genes constitute diverse functional groups

Among the new candidate longevity genes that are annotated with a possible function (~75%, 66/89) (Table 1; Supplementary Fig. 1), a large number (~25%, 17/66) are involved in some aspect of metabolism, such as carbohydrate metabolism, alcohol metabolism, citric acid cycle, oxidative phosphorylation, and purine metabolism (Table 1). Although it is not clear how RNAi inactivation of each of the enzymes in these different metabolic processes regulates lifespan, an attractive hypothesis is that reduced function of many of these enzymes decreases the tempo or mode of energy generation, which might regulate longevity through mechanisms similar to that of caloric restriction.

Among the longevity genes predicted to participate in energy metabolism, >50% correspond to subunits of the ETC (complex I: D2030.4, K04G7.4, T20H4.5; complex IV: F26E4.6, F26E4.9, W09C5.8; complex V: C53B7.4.). Although complex III subunit was not found in our current screen, we previously showed that RNAi inactiva-

tion of the complex III subunit T02H6.11 could extend lifespan (Lee et al. 2003b). Complex II subunit has not been identified in any of our screens. Complex II is made up of a relatively small number of subunits and may represent a smaller target size for our screen. We hypothesize that reduced function of the different ETC components regulates lifespan through a common molecular mechanism, probably by lowering ATP generation and/or free radical production. Indeed, four of the RNAi inactivations identified here were characterized previously to induce lower ATP production and pleiotropic phenotypes, including small body size, slow pumping, slow growth rate, and sterility (Dillin et al. 2002; Lee et al. 2003b). We analyzed the three additional ETC subunits identified in the current screen and found that they also induced growth rate and reproduction defect (Supplementary Table 3). Interestingly, when worms are exposed to RNAi prior to embryogenesis, a much more severe growth phenotype was detected (Supplementary Table 3). Oxidative phosphorylation is required for *C. elegans* survival; lifespan extension is likely caused by partial reduction of mitochondrial electron transport activity, as severe reduction of ETC function causes larval arrest and lethality (Supplementary Table 3; Tsang and Lemire 2003).

Nuclear genes that are essential to mitochondrial function are not fully described in the *C. elegans* genome. To assess which of the 90 RNAi inactivations regulate mitochondrial function, we used the reporter *hsp-6*::*gfp(zcIs13)*, which encodes a mitochondrial chaperone (Yoneda et al. 2004), to monitor a mitochondrial unfolded protein response. As expected, RNAi inactivation of the candidate genes predicted to function in the mitochondria, such as ETC subunits (D2030.4, K04G7.4, T20H4.5, F26E4.6, F26E4.9, W09C5.8, C53B7.4) and aconitase (F54H12.1), induced *hsp-6*::GFP expression (Fig. 1). ETC components work as large protein complexes; RNAi inactivation of one subunit will likely interfere with assembly of the entire protein complex and cause accumulation of unfolded proteins in the mitochondria (Yoneda et al. 2004). Interestingly, in addition to the candidate genes that are annotated to be mitochondrial, we found RNAi inactivation of four additional longevity genes [C39F7.2, Y53F4B.23, Y75B8A.33, Y92C3A.1(*cdh-12*)] also induced *hsp-6*::GFP, albeit to a lesser degree than RNAi of ETC subunits (Fig. 1). These results suggest that these four candidate genes might also function in the mitochondria or be necessary for mitochondrial biogenesis. Two of these genes encode proteins of unknown function (Y53F4B.23, Y75B8A.33), whereas one of them encodes a protein predicted to contain various protein–protein interaction domains (C39F7.2), and the remaining one encodes a cadherin homolog (*cdh-12*). The cadherin domain is usually present in proteins required for cell–cell junction formation. It is curious that *cdh-12* RNAi inactivation should produce a mitochondrial unfolded protein response. Blastn analysis indicates that the RNAi construct Y119D3_451.a clearly targets gene *cdh-12*, but it also displays longer than 20-bp sequence homology to a number of other *C. elegans* genes, and may knockdown expression of additional gene(s). It is also important to note that for the genes that do not function as large protein complexes in the mitochondria, RNAi inactivations are not expected to induce a robust misfolded protein response, and will not be detected by a survey using the *hsp-6*::*gfp* reporter. Therefore, it is very possible that additional candidate longevity genes recovered from our screen function in mitochondria.

In addition to oxidative phosphorylation components, the longevity RNAi screens identified several enzymes required for carbohydrate metabolism (e.g., UTP-glucose-1-phosphate uridylyltransferase K08E3.5), as well as the citric acid cycle (e.g., aconitate hydratase F54H12.1, isocitrate dehydrogenase F43G9.1 and F59B8.2), that regulate *C. elegans* lifespan. Carbohydrate metabolism and citric acid cycle are processes important to generating fuel necessary for oxidative phosphorylation. One interesting speculation is that RNAi of the carbohydrate metabolic enzymes and the citric acid cycle genes, extend lifespan via mechanisms similar to that of reduced oxidative phosphorylation.

On the other hand, it is also possible that RNAi inactivation of some of the specific metabolic enzymes block particular pathways, leading to elevated or reduced levels of certain metabolic intermediates that might be especially important for longevity.

A number of the longevity genes are annotated to participate in signal transduction. For instance, in addition to *akt-1*, two additional candidate longevity genes (C34B4.1, K10B4.3) encoding proteins with PH domains were identified. A well-established function of the PH domain is the binding of phosphatidylinositol lipids; an intriguing possibility is that these two candidate genes might respond to *age-1* signaling to regulate *C. elegans* lifespan. Interestingly, RNAi of C34B4.1 or K10B4.3 required *daf-16* to increase lifespan, suggesting that they act upstream of *daf-16* (see below; Supplementary Table 1).

Four RNAi constructs identified in our screen inactivate annotated G-protein coupled receptors (GPCR) (T04A11.10, T05E12.4, T26H5.1, Y46H3C_13.a). Although RNAi construct Y46H3C_13.a might target genes Y46H3C.1 and Y46H3C.2, both of the probable target genes are annotated to be GPCRs. In *C. elegans*, GPCRs often act as chemosensory receptors, but many are predicted to be neuropeptide receptors as well (Bargmann 1998). Sensory neurons regulate *C. elegans* lifespan (Apfeld and Kenyon 1999); it is possible that some of the GPCRs we identified participate in sensory perception and transmit environmental cues to downstream signaling pathways. For example, production of various insulins in these sensory neurons might regulate longevity. However, because feeding RNAi is thought to work inefficiently in *C. elegans* neurons, neuronal-specific genes are unlikely to be recovered in our screen. On the other hand, GPCRs have a wide range of substrates. Recently, citric acid cycle intermediates have been identified to bind to GPCRs and trigger downstream signaling (He et al. 2004). This is especially intriguing, consid-

Table 1. *A genome-wide screen identified 90 RNAi inactivations that induce lifespan extension*

Primer name	Gene name	Brief description	Functional group	Fly ortholog	Mouse ortholog	Human ortholog
F07A5.1	F07A5.1	Innexin, required for GAP junction	Cell structure			
F35C8.6	F35C8.6	Profilin	Cell structure			
K07H8.1	K07H8.1	Tubulin-specific chaperone	Cell structure	yes	yes	yes
W05B2.5	W05B2.5	Collagen	Cell structure			
Y119D3_451.a	Y92C3A.1	Cadherin domain	Cell structure			
C35A11.3	C35A11.3	Mucin	Cell surface			
F08H9.5	F08H9.5	C-type lectin	Cell surface			
K11D12.1	K11D12.1	Mucin	Cell surface			
ZK896.7	ZK896.7	C-type lectin	Cell surface			
C13C4.2	C13C4.2	Nuclear hormone receptor	Gene expression			
F15E6.1	F15E6.1[a]	SET domain; PHD-finger	Gene expression		yes	
F54C4.2	F54C4.2	Transcription elongation factor Spt4	Gene expression	yes	yes	yes
R11E3.4	R11E3.4	SET domain	Gene expression			
T01B10.4	T01B10.4	Nuclear hormone receptor	Gene expression			
T09A5.8	T09A5.8	Chromo domain	Gene expression			
ZC64.3	ZC64.3	Homeobox protein ceh-18	Gene expression			
T05A1.4	T05A1.4	Retrotransposon integrase	Integrase			
D1054.8	D1054.8	Glucose/ribitol dehydrogenase	Metabolism			
W09H1.5	W09H1.5	Zinc-containing alcohol dehydrogenase superfamily	Metabolism	yes	yes	yes
K08E3.5	K08E3.5	UTP-glucose-1-phosphate uridylyltransferase	Metabolism: galactose/ starch/sucrose metabolism	yes	yes	yes
F57B10.3	F57B10.3	Phosphoglycerate mutase	Metabolism: glycolysis			
F43G9.1	F43G9.1	Isocitrate dehydrogenase	Metabolism: TCA cycle	yes	yes	yes
F54H12.1	F54H12.1	Aconitase	Metabolism: TCA cycle	yes	yes	yes
F59B8.2	F59B8.2	Isocitrate dehydrogenase	Metabolism: TCA cycle	yes	yes	yes
B0261.4	B0261.4	Mitochondrial 39-S ribosomal protein L47	Metabolism: ETC	yes	yes	yes
C33F10.12	C33F10.12	Mitochondrial carrier	Metabolism			
C53B7.4	C53B7.4	Mitochondrial ATP synthase g subunit	Metabolism: ETC	yes		
D2030.4	D2030.4	NADH:CoQ oxidoreductase subunit B18	Metabolism: ETC	yes	yes	yes
F26E4.6	F26E4.6	Cytochrome C oxidase	Metabolism: ETC			
F26E4.9	F26E4.9	Cytochrome C oxidase	Metabolism: ETC	yes	yes	yes
K04G7.4	K04G7.4	NADH dehydrogenase	Metabolism: ETC	yes	yes	yes
T20H4.5	T20H4.5	NADH-quinone oxidoreductase	Metabolism: ETC	yes	yes	yes
W09C5.8	W09C5.8	Cytochrome c oxidase subunit IV	Metabolism: ETC	yes	yes	yes
F55B11.1	F55B11.1	Xanthine Dehydrogenase (XDH)	Metabolism: purine metabolism	yes	yes	yes
Y71G12A_202.a	Y71G12B.4	Peptidylglycine alpha-amidating monooxygenase	Neuropeptide synthesis	yes		
W01A11.3	W01A11.3	Spectrin repeat	Nuclear migration			
C08B11.7	C08B11.7	Ubiquitin C-terminal hydrolase	Protein turnover	yes	yes	yes
C16C10.7	C16C10.7	Ubiquitin E3 ligase	Protein turnover	yes	yes	yes
C51E3.7	C51E3.7	egl-3 prohormone convertase	Protein turnover	yes	yes	yes
F57C12.1	F57C12.1	Astacin Peptidase	Protein turnover			
T05G5.10	T05G5.10	Eukaryotic initiation factor 5A iff-1	Protein turnover	yes		
Y71H2_380.a	Y71H2AR.2	Papain family cysteine protease	Protein turnover			
C01F6.7	C01F6.6	PDZ domain	Protein–protein interaction			
F23B2.1	C01F6.6	PDZ domain	Protein–protein interaction			
Y50D4A_25.a	C39F7.2	Zinc (RING) finger, SPRY domain, Fibronectin type III domain	Protein–protein interaction	yes	yes	yes
F09F7.5	F09F7.5	P21-Rho-binding domain	Protein–protein interaction			
F21H12.1	F21H12.1	WD domain	Protein–protein interaction	yes	yes	yes
Y39H10A_224.b	Y39H10A.6	WD domain, C-terminal to LisH motif	Protein–protein interaction	yes	yes	yes
Y54E5A.7	Y54E5A.7	SPRY domain, C-terminal to LisH motif	Protein–protein interaction	yes	yes	yes
C36H8.1	C36H8.1	MSP (Major sperm protein)	Reproduction			
C39E9.1	C39E9.1	Testis-specific protein TPX-1 like	Reproduction			
Y39F10C.1	Y39F10C.1	Vitelline membrane outer layer protein I (VOMI)	Reproduction			
B0334.8	B0334.8	age-1 Phosphatidylinositol 3- and 4-kinase	Signaling	yes	yes	yes
C01F6.1	C01F6.1	Copine	Signaling			
C04H5.3	C04H5.3	Adenylate and Guanylate cyclase, natriuretic peptide receptor	Signaling			
C12D8.10	C12D8.10	akt-1 serine/threonine kinase	Signaling	yes	yes	yes
C27B7.7	C27B7.7	Ig domain, Fibronectin type III domain	Signaling			
C34B4.1	C34B4.1	PH domain	Signaling	yes	yes	yes

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Table 1. *(continued)*

Primer name	Gene name	Brief description	Functional group	Fly ortholog	Mouse ortholog	Human ortholog
F35D2.3	F35D2.3	EGF-like	Signaling			
K08D10.7	K08D10.7	Scramblase	Signaling			
K10B4.3	K10B4.3	PH domain	Signaling	yes	yes	yes
T04A11.10	T04A11.10	7TM receptor	Signaling			
T05E12.4	T05E12.4	7TM chemoreceptor, srw family	Signaling			
T26H5.1	T26H5.1	7Tm receptor	Signaling			
Y46H3C_13.a	Y46H3C.1/.2[b]	7TM chemoreceptor, srw family	Signaling			
F40F8.5	F40F8.5	ABC transporter	Small molecule transport			
C09B7.2	C09B7.2	Transposase	Transposase			
C26B2.2	C26B2.2		unknown			
C32H11.1	C32H11.1	Domain of unknown function DUF141	unknown			
C32H11.5	C32H11.5[c]	Domain of unknown function DUF148	unknown			
D2054.8	E03H12.5		unknown			
F45H10.4	F45H10.4		unknown			
F49F1.12	F49F1.12		unknown			
F55B11.3	F55B11.5/.3[d]		unknown			
H06H21.8	H06H21.8	Domain of unknown function DUF227	unknown			
K10D2.2	K10D2.2	PAP/25A associated domain	unknown			
R05A10.5	R05A10.5		unknown			
R08E3.3	R08E3.3		unknown	yes	yes	yes
T06G6.4	T06G6.4		unknown			
T07A9.8	T07A9.8		unknown	yes	yes	yes
Y43F8B.12	Y43F8B.12		unknown			
Y43H11_160.a	Y43h11AL.2		unknown		yes	yes
Y46H3C_14.b	Y46H3C.6		unknown			
Y53F4C.d	Y53F4B.23		unknown			
Y56A3A.9	Y56A3A.9		unknown			
Y75B8A.13	Y75B8A.13		unknown			
Y75B8A.33	Y75B8A.33		unknown			
ZK520.2	ZK520.2		unknown			
C18E9.4	C18E9.4/.10[e]					
Y37D8A.12	Y37D8A.12[f]					

[a]F15E6.1 RNAi clone may hybridize to both F15E6.1 and Y51H4A.12, two PHD domain-containing proteins.
[b]Y46H3C_13.a RNAi clone spans both Y46H3C.1 and Y46H3C.2, two highly similar genes coding for 7TM chemoreceptor in the srw family.
[c]C32H11.5 RNAi clone may hybridize to multiple genes (Kamath et al. 2003); C32H11.5; C32H11.6; C32H11.8; C32H11.11; all of which contain DUF148.
[d]F55B11.3 RNAi clone may hybridize to both F55B11.5 and F55B11.3, two highly similar genes of unknown function.
[e]C18E9.4 RNAi clone spans both C18E9.4, which encodes a NADH-ubiquinone oxidoreductase, and C18E9.10, which encodes an unknown protein.
[f]Y37D8A.12 RNAi clone may hybridize to multiple genes (Kamath et al. 2003).

ering that our results indicate that RNAi inactivation of citric cycle enzymes promote *C. elegans* longevity.

Other signaling proteins recovered in our screen include a copine protein (C01F6.1), an evolutionarily conserved family of proteins that binds phospholipids in a Ca^{2+}-dependent manner (Tomsig and Creutz 2002), and a guanylate cyclase (C04H5.3) that might act as a natriuretic receptor. Furthermore, a number of the longevity genes encode proteins that contain domains that might act as cell-surface receptors, such as EGF-like domains (F35D2.3) and Ig domains (C27B7.7). Lastly, a few longevity regulatory genes are predicted to encode scaffolding proteins, containing multiple protein–protein interaction motifs, such as WD domains (F21H12.1, Y39H10A.6), Zinc/RING fingers (C39F7.2), or PDZ domains (C01F6.6). These findings together suggest that these candidate longevity genes might participate in various signaling pathways. Given the importance of insulin-like signaling in lifespan in diverse species, it will be important to find new components of the insulin-like signaling pathway, as well as genes that might act in novel signaling pathways to regulate longevity.

Our screen revealed that RNAi of *iff-1* (T05G5.10), a *C. elegans* homolog of the translation initiation factor eIF-5A, extends lifespan. Interestingly, protein synthesis has been suggested to regulate longevity in other animals. In the long-lived Snell dwarf mouse, translation initiation is decreased compared with control (Hsieh and Papaconstantinou 2004). Reduced translation initiation is proposed to be a consequence of reduced insulin and mTOR signaling in these long-lived mice.

In addition, several genes likely involved in protein processing/degradation were also identified in our screen. These include several proteases (C51E3.7, F57C12.1, Y71H2AR.2), an ubiquitin ligase (C16C10.7), and an ubiquitin C-terminal hydrolase (C08B11.7), which functions to remove ubiquitin from modified proteins. Among the proteases, one particularly interesting candidate gene is *egl-3* (C51E3.7), which encodes a prohormone convertase (Kass et al. 2001). Prohormone convertases mediate the proteolytic processing of various hormones and neural peptides, including insulin. It is possible that RNAi of *egl-3* extends lifespan by inactivating the processing of certain insulin(s). It is interest-

Table 2. *A subset of RNAi inactivations were analyzed for genetic interactions with* daf-16 *and* sir-2.1

				rrf-3 lifespan at 20°C					Genetic interactions[f]	
Chr.	Gene name	Brief description	Functional group	Mean[a]	Std err[b]	Median 25%[c]	Total (cens)[d]	*p*-value[e]	*daf-16; rrf-3*[g]	*sir-2.1; rrf-3*[h]
2	B0334.8	age-1 phosphatidylinositol 3- and 4-kinase	Signaling	31.51	0.76	33/36	86 (4)	<0.0001	not extend	extend
5	C12D8.10	akt-1 serine/threonine kinase	Signaling	30.68	0.65	31/33	77 (6)	<0.0001	not extend	extend
3	K08E3.5	UTP-glucose-1-phosphate uridylyltransferase	Metabolism	22.84	0.50	24/26	74 (10)	<0.0001	extend	extend
3	K04G7.4	NADH dehydrogenase	Metabolism: ETC	22.76	0.46	24/26	63 (5)	0.0008	extend	extend
5	Y39H10A.6	WD domain, C-terminal to LisH motif	Protein–protein interaction	22.38	0.39	22/24	94 (11)	<0.0001	not extend	extend
5	Y46H3C.1/.2	7TM chemoreceptor, srw family	Signaling	21.95	0.45	22/26	98 (21)	<0.0001	not extend	extend
2	F21H12.1	WD domain	Protein–protein interaction	21.84	0.31	22/24	105 (6)	<0.0001	not extend	not extend
3	Y75B8A.33		unknown	21.68	0.86	20/24	25 (5)	0.0008	not extend	extend
X	ZC64.3	Homeobox protein ceh-18	Gene expression	21.57	0.37	22/24	112 (16)	<0.0001	extend	not extend
1	F07A5.1	Innexin, required for GAP junction	Cell structure	21.56	0.40	22/24	87 (8)	<0.0001	not extend	extend
3	F54C4.2	Transcription elongation factor Spt4	Gene expression	21.56	0.48	20/26	83 (12)	<0.0001	extend	extend
4	F15E6.1	SET domain; PHD-finger	Gene expression	21.25	0.40	20/24	96 (7)	<0.0001	not extend	extend
4	F55B11.1	Xanthine Dehydrogenase (XDH)	Metabolism	21.05	0.44	20/24	107 (27)	<0.0001	not extend	not extend
X	C09B7.2	Transposase	Transposase	21.03	0.38	20/24	100 (20)	<0.0001	not extend	extend
4	C32H11.1	Domain of unknown function DUF141	unknown	20.89	0.37	20/22	82 (9)	<0.0001	not extend	extend
5	K11D12.1	Mucin	Cell surface	20.84	0.36	20/22	94 (10)	<0.0001	not extend	extend
3	W05B2.5	Collagen	Cell structure	20.80	0.40	20/24	92 (15)	<0.0001	not extend	extend
2	Y43H11AL.2		unknown	20.72	0.55	20/24	58 (6)	0.0007	not extend	extend
2	Y39F10C.1	Vitelline membrane outer layer protein I (VOMI)	Reproduction	20.70	0.44	20/24	94 (10)	0.0002	not extend	extend
X	R08E3.3		unknown	20.66	0.31	20/22	103 (27)	<0.0001	extend	extend
3	T05G5.10	Eukaryotic initiation factor 5A iff-1	Protein turnover	20.61	0.37	20/22	103 (14)	<0.0001	not extend	extend
4	K08D10.7	Scramblase	Signaling	20.53	0.39	20/22	91 (7)	0.0005	not extend	not extend
1	Y54E5A.7	SPRY domain, C-terminal to LisH motif	Protein–protein interaction	20.53	0.42	20/22	77 (3)	0.0006	extend	extend
4	C01F6.1	Copine	Signaling	20.37	0.51	20/24	78 (9)	0.0007	not extend	not extend
4	C39E9.1	Testis-specific protein TPX-1 like	Reproduction	20.28	0.52	20/24	86 (14)	0.0001	not extend	not extend
4	R05A10.5		unknown	20.04	0.36	20/22	90 (6)	0.0002	not extend	not extend
4	C36H8.1	MSP (Major sperm protein)	Reproduction	20.04	0.44	20/22	62 (2)	0.0185	not extend	not extend
3	F09F7.5	P21-Rho-binding domain	Protein-protein interaction	20.01	0.42	20/22	81 (9)	0.0126	extend	not extend
5	C39F7.2	Zinc (RING) finger, SPRY domain, Fibronectin type III domain	Protein–protein interaction	20.00	0.45	20/22	64 (9)	0.0008	not extend	extend
3	Y56A3A.9		unknown	19.96	0.35	20/22	80 (5)	0.0246	not extend	extend
1	T06G6.4		unknown	19.95	0.47	20/22	66 (5)	0.0127	not extend	extend
3	T20H4.5	NADH-quinone oxidoreductase	Metabolism: ETC	19.85	0.56	20/24	62 (4)	0.0016	extend	extend
3	Y75B8A.13		unknown	19.79	0.34	20/22	108 (16)	0.0219	not extend	not extend
3	Y71H2AR.2	Papain family cysteine protease	Protein turnover	19.73	0.48	20/22	70 (5)	0.0461	not extend	not extend
3	F54H12.1	Aconitase	Metabolism	19.69	0.43	20/22	65 (10)	0.0101	extend	extend
	L4440 control[i]			18.68	0.35	18/20	130 (37)			
	L4440 control[i]			18.40	0.26	18/20	142 (24)			

[a]The mean lifespan is shown in days.
[b]The standard error of the mean is shown in days.
[c]The median lifespan and the time when 25% of individuals remain alive are shown in days.
[d]The total number of individuals scored is shown (followed by the number of individuals censored due to protruding/bursting vulva, bagging, or crawling off the agar).
[e]The *p*-value from a log rank test comparing RNAi treatment populations to the control population is shown.
[f]Suppression of RNAi-induced extended lifespan in short-lived mutant strains.
[g]The effect of RNAi feeding on lifespan using a *daf-16(mgDf47);rrf-3(pk1426)* mutant strain is summarized.
[h]The effect of RNAi feeding on lifespan using a *sir-2.1(ok434;rrf-3(pk1426)* mutant strain is summarized.
[i]The lifespan results of two control populations fed the L4440 empty vector, which were used in comparison with the above RNAi treatments.

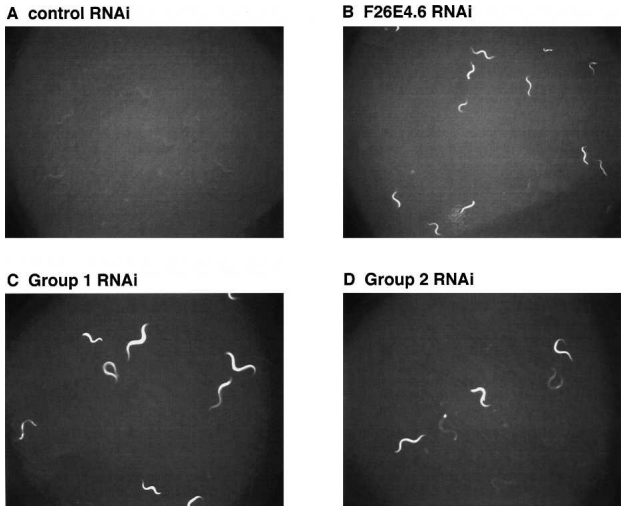

Figure 1. RNAi inactivation of multiple candidate longevity genes induces *hsp-6*::GFP expression. (*A*) Basal levels of *hsp-6*::GFP expression in worms treated with control RNAi (L4440 empty vector). (*B*) Greatly enhanced *hsp-6*::GFP expression in worms treated with RNAi targeting F26E4.6 (cytochrome C oxidase subunit) (Yoneda et al. 2004). (*C*) A representative image of enhanced *hsp-6*::GFP expression in worms treated with a "group 1" RNAi. Group 1 RNAi include F54H12.1, C53B7.4, D2030.4, F26E4.6, F26E4.9, K04G7.4, T20H4.5, and W09C5.8. (*D*) A representative picture of somewhat enhanced *hsp-6*::GFP expression in worms treated with a "group 2" RNAi. Group 2 RNAi include B0261.4, C39F7.2, Y53F4B.23, Y75B8A.33, and Y92C3A.1.

ing to note that *egl-3* RNAi required *daf-16* activity to prolong lifespan (see below; Supplementary Table 1), suggesting that *egl-3* acts upstream of *daf-16*.

A number of the candidate longevity genes are predicted to encode proteins that might regulate gene expression. These include proteins with motifs that might modify chromatin structures (such as PHD domains, SET domains, or chromo domains) (F15E6.1, R11E3.4, T09A5.8), established transcription factors (such as SPT-4 F54C4.2, CEH-18 ZC64.3), and annotated nuclear hormone receptors (NHR) (C13C4.2, T01B10.4). Similar to that in yeast, SIR-2.1 may regulate *C. elegans* lifespan by modifying global or local chromatin structures. Some of the chromatin factors we identified might cooperate with *sir-2.1* to modulate longevity. Furthermore, the NHR DAF-12 acts downstream of DAF-16 to modulate lifespan and dauer formation. It will be very interesting to test whether some of the NHRs identified here might functionally interact with DAF-12.

Additional longevity regulatory genes include several proteins likely involved in reproduction, such as a vitelline membrane outer layer protein (Y39F10C.1), a major sperm protein (C36H8.1), and a testis-specific TPX protein (C39E9.1). A prominent theory of aging postulates that the vast amount of energy an animal spends on reproduction will limit the resources remaining for life maintenance. RNAi inactivation of these genes may cause lifespan extension by reducing energy expenditure through reproduction. However, it is important to note that many examples counter to this theory exist (Finch 1990), and many sterile mutants in *C. elegans* show normal lifespan.

We also identified an annotated transposase (C09B7.2) and a possible retrotransposon integrase (T05A1.4) in our screen. In a recent genome-wide expression profiling of aging *C. elegans* (Lund et al. 2002), transposases are among the groups of genes whose expression is elevated during aging. Increased transposase activity might cause increased transposon hopping and genomic instability. It is possible that RNAi inactivation of one or more transposases, and/or retrotransposon integrases, might extend lifespan by increasing genomic stability.

To determine the comprehensiveness of our genome-wide screen, we compared the longevity genes we identified with the representative longevity genes that have been reported previously. In the *daf-2*/insulin signaling pathway, reduction in *daf-2*, *age-1*, *pdk-1*, *akt-1*, *sgk-1*, and *ins-18* gene activities extend lifespan. Of these genes, *age-1*, *akt-1*, *daf-2*, and *ins-18* are represented in the 80% coverage RNAi library. Three of these four genes, *age-1*, *akt-1*, and *daf-2*, emerged in the high-throughput screen without reference to their identity, and two of these, *age-1* and *akt-1*, were intense enough to pass the secondary screens. The *daf-2* RNAi clone did not pass our subsequent multiple rounds of retest, probably because of its weaker phenotype (~20% lifespan extension induced by the particular *daf-2* RNAi clone in the library vs. ~50% lifespan extension induced by *age-1* or *akt-1* RNAi) and the variability of RNAi. We did not detect a lifespan phenotype with *ins-18* RNAi. This is not surprising, as *ins-18* RNAi was capable of extending lifespan only upon multiple generations of feeding inactivation (Kawano et al. 2000), a strategy that is very different from our high-throughput RNAi strategy.

Previous genetic screens identified mitochondrial genes that affect *C. elegans* lifespan were as follows: *clk-1*, *isp-1*, and *lrs-2* (Lakowski and Hekimi 1996; Feng et al. 2001; Lee et al. 2003b). Only the *clk-1* RNAi construct is present in the RNAi library, and we were not able to detect a lifespan phenotype associated with *clk-1* RNAi. RNAi inactivation of the mitochondrial genes *atp-3* (F27C1.7), *nuo-2* (T10E9.7), *cyc-1* (C54G4.8), *cco-1* (F26E4.9), D2030.4, T02H6.11, F26E4.6, W09C5.8, B0261.4, T06D8.6, F13G3.7, and K01C8.7 (Dillin et al. 2002; Lee et al. 2003b), have previously been reported to extend lifespan. Eight of these 12 genes were recovered in our high-throughput screens, and five were among the final 89 candidate longevity genes: *cco-1* (F26E4.9), D2030.4, F26E4.6, W09C5.8, and B0261.4 (Table 1). In addition, our genome-wide screen identified seven additional mitochondrial function genes that can affect lifespan (F43G9.1, F54H12.1, F59B8.2, C33F10.12, C53B7.4, K04G7.4, and T20H4.5) (Table 1).

Previous forward genetic screens also revealed that loss-of-function mutations of a number of genes important for sensory neuron development extend lifespan (Apfeld and Kenyon 1999). Our RNAi screen did not recover any gene that is implicated to affect sensory neu-

ron development. This is not surprising, considering that feeding RNAi does not work well in neurons, and we have expected our screen to miss the group of longevity genes that specifically function in the nervous system.

The eating defective (*eat*) mutants were specifically examined for a lifespan phenotype to show that caloric restriction also extends lifespan in *C. elegans* (Lakowski and Hekimi 1998). Among the long-lived *eat* mutants, *eat-1* (T11B7.4), *eat-3* (D2013.5), and *eat-6* (B0365.3) RNAi are represented in the library, but were not identified in our screen. However, we did recover *eat-4* RNAi in our high-throughput screens (among the 600 candidates). Our subsequent analyses indicate that the *eat-1*, *eat-3*, or *eat-6* RNAi constructs did not significantly prolong lifespan under our RNAi conditions.

Loss-of-function mutation of *glp-1* results in loss of germline stem cells and extended lifespan (Arantes-Oliveira et al. 2002). Although *glp-1* RNAi is represented in the RNAi library, our analyses showed that the *glp-1* RNAi construct was not able to induce lifespan extension, probably because our feeding RNAi approach does not deplete *glp-1* early enough to ablate the germline stem cells.

Finally, from a microarray experiment focusing on identifying possible DAF-16 downstream genes, Murphy et al. (2003) showed that RNAi inactivation of ~20 putative DAF-16 downstream genes resulted in a lifespan increase that ranged from 3% to 55%. Because of the modest lifespan extension caused by some of these RNAi inactivations, we do not expect to recover many of these 20 genes in our screen. Interestingly, F55G11.5 and *gcy-18* (ZK896.8) were among the 600 candidates we identified in our high-throughput screenings. Furthermore, we identified additional candidate genes that may participate in functions similar to some of the DAF-16 downstream genes, and may affect lifespan via similar mechanisms. For example, we identified *gcy-23* in our screen, which may affect similar cellular processes as *gcy-18* (Murphy et al. 2003). Although we did not recover the DAF-16 downstream genes *vit-2* and *vit-5* (vitellogenin) in our screen (Murphy et al. 2003), we did recover a vitellogenin membrane outer layer protein (Y39F10C.1). Lastly, although we did not recover the DAF-16 downstream genes C32H11.10 and C32H11.12 (Murphy et al. 2003), both encoding DUF141 proteins, we did recover five different genes predicted to encode DUF141 proteins from our high-throughput screenings, and the DUF141 encoding gene C32H11.1 exhibited a robust and consistent phenotype and was among the final 89 longevity candidate genes (Table 1).

Our comparison indicates that although we attempted to be comprehensive, our genome-wide screen is not saturating. This is likely due to the intrinsic limitations that feeding RNAi tends to be variable and often only gives a hypomorphic phenotype. Furthermore, our high-throughput feeding RNAi strategy exposed animals to RNAi starting as L1s, which might not be optimal depending on the temporal requirement of the longevity genes. It is important to note that our RNAi screen represents an unbiased screening strategy, and our screen is very successful in recovering many of the known longevity genes identified through previous unbiased screens (e.g., insulin-signaling components, mitochondrial genes). The hit rate for our screen regarding the other longevity genes identified through candidate gene approach is lower (e.g., *eat* genes). This is likely because the lifespan phenotype associated with these genes is modest and is easily missed in high-throughput screenings. Although our screen did not recover all of the previously reported longevity genes, very often, our screen revealed candidate longevity genes that are predicted to participate in functions similar to that of the longevity genes reported previously. This indicates that our screen appears to have targeted most of the gene classes that, when inactivated, can promote longevity, and suggests that our genome-wide RNAi screen is valuable in providing a global view of many of the gene classes that normally limit lifespan.

Genomic classification of longevity genes

Some of the longevity genes recovered from our screen were closely located on the chromosomes. For instance, four different cosmids were found to each contain at least two candidate longevity genes (C01F6.1 and C01F6.6, C32H11.1 and C32H11.11, F26E4.6 and F26E4.9, F55B11.1 and F55B11.11). We suspect that some of these candidate genes might be different genes of the same operon, and their RNAi might affect all of the genes on the same operon and cause a common phenotypic outcome. However, a close inspection of each of the genomic regions did not reveal that any of the genes listed above might constitute part of an operon (Blumenthal et al. 2002). Therefore, the fact that some of the candidate longevity genes are closely located might be due to their related function on longevity control or might simply be coincidence.

We attempted to classify the candidate longevity genes by examining whether they share any similar expression pattern. Using the TopoExpression Mountains characterized by Kim et al. (2001), we found that the candidate longevity genes fit into many different expression groups. Interestingly, five expression mountains are significantly overrepresented ($P < 0.001$) (Supplementary Table 2). These include the mountains enriched in genes involved in biosynthesis, carbohydrate metabolism, energy generation, mitochondrial, and protein expression/energy generation, respectively. Further inspection reveals that a similar subset of candidate genes, the ones annotated with a metabolic function (Table 1), fit into each of these five different mountains. Over-representation of these five expression mountains among our candidate longevity genes is consistent with our finding that energy metabolism represents a major gene class important for *C. elegans* lifespan.

We also used the genome-wide protein–protein interaction maps published by Li et al. (2004) to examine whether some of the candidate longevity genes might participate in common protein complexes. Two-hybrid protein–protein interaction data are available for only 25

of the 89 candidate genes. Among these 25 predicted proteins, no direct protein–protein interaction was detected in the two-hybrid screen (Li et al. 2004). However, several of the candidate genes identified in our screen might form a complex with each other through one bridging protein (Supplementary Fig. 2). We did not look for complex formation between the candidates that might be mediated by more than one bridging protein, as such a survey revealed complicated interaction maps that are difficult to interpret. Although each pair-wise interaction detected in the two-hybrid screen might occur individually under different circumstances, it is also possible that the linked interactions detected by the genomic two-hybrid screen suggest that some of the candidate longevity genes might function as protein complexes and cooperate to influence lifespan. It will be interesting to test whether RNAi inactivation of the two-hybrid interactors of the candidate longevity genes might also cause a lifespan phenotype in future investigations.

Dauer analysis

Because the prominent longevity pathway *daf-2*/insulin-like signaling is also critical for controlling dauer formation, we sought to determine whether any of the new longevity candidate genes might also affect dauer formation. Feeding RNAi in general is not effective in inducing dauer formation, probably because it does not efficiently knockdown neuronal genes. We utilized a sensitive assay in which we examined *daf-2(e1370)* worms for an ability to recover from dauer arrest at the semi-nonpermissive temperature 22°C (Lee et al. 2003a; Murphy et al. 2003). We surveyed the 90-candidate longevity RNAi. Although the survey was done blind, we successfully identified *age-1* and *akt-1* RNAi to enhance the dauer formation phenotype of *daf-2(e1370)* mutant (Supplementary Table 4). We additionally found that RNAi of K08E3.5 and F15E6.1 moderately enhanced the dauer formation phenotype, suggesting that these candidate genes might have roles in both longevity and dauer development (Supplementary Table 4). Interestingly, RNAi of most of the ETC genes also enhanced the dauer phenotype. This is likely due to the slower growth of the RNAi animals, which would delay dauer exit and act like an enhancer in our assay. Alternatively, these animals might be hypometabolic and might be dauer prone.

Genetic interaction of longevity genes

One method of organizing the long lists of longevity regulators into pathways is by genetic interaction analysis with other known longevity mutants. Therefore, we tested whether RNAi of a subset of the candidates might require *daf-16* or *sir-2.1* activity to regulate lifespan by performing RNAi lifespan experiments using either a *daf-16* deletion or a *sir-2.1* deletion mutant strain. To ensure that the different genetic backgrounds do not affect the efficiency of feeding RNAi, we used several test RNAi clones that produce visible phenotypes (e.g., *dpy-13*, *hmr-1* [Kennedy et al. 2004]) and demonstrated that the *rrf-3(pk1426);daf-16(mgDf47)* and *rrf-3(pk1426);sir-2.1(ok434)* mutants exhibited similar *dpy-13* and *hmr-1* phenotypes compared with *rrf-3(pk1426)* (data not shown). We have thus far tested the longevity RNAi candidates that exhibited the most robust phenotypes, those with a P-value ≤ 0.001 in the lifespan experiments using *rrf-3* mutant at 25°C (Supplementary Table 1). A total of 35 candidate longevity genes were chosen for our genetic interaction analyses, and they fall into functional groups that display a distribution similar to that of the entire set of candidate genes (Table 2).

It is interesting to note that the *rrf-3(pk1426);sir-2.1 (ok434)* mutant displayed a slightly shorter lifespan compared with *rrf-3(pk1426)*, an observation that is consistent with what is known about Sir2 deletion in yeast. However, the lifespan shortening of *rrf-3(pk1426);sir-2.1 (ok434)* is not as dramatic as that of the *rrf-3(pk1426); daf-16(mgDf47)* mutant (Supplementary Table 1). Our results indicate that *daf-16* and *sir-2.1* show overlapping, yet distinct genetic interactions with the panel of candidate longevity genes (Table 2; Supplementary Table 1). Of the 35 candidate genes tested, RNAi inactivation of 26 of them requires *daf-16* activity to extend lifespan, whereas RNAi of 11 candidates requires *sir-2.1* activity to extend lifespan. Furthermore, whereas a number of candidate genes require both *daf-16* and *sir-2.1* activity to extend lifespan (nine candidates), a number of candidate genes require only *daf-16* (17 candidates) or only *sir-2.1* (two candidates) activity to regulate lifespan (Table 2). These results suggest that *daf-16* and *sir-2.1* cooperate to regulate lifespan in some instances. However, both *daf-16* and *sir-2.1* have significant independent activity that is important for lifespan regulation.

Mammalian SIRT1 affects gene expression through modification of multiple chromatin proteins; *C. elegans* SIR-2.1 may similarly modify multiple substrates. Loss of *daf-16* suppresses the lifespan extension caused by overexpression of SIR-2.1 (Tissenbaum and Guarente 2001), indicating that *daf-16* is a major output of *sir-2.1*. Our analyses suggest that *sir-2.1* is likely to have additional downstream mediators that also regulate longevity. In addition, both *age-1* and *akt-1* RNAi robustly extend the lifespan of *sir-2.1* deletion mutant animals. These results indicate that, even in the case that *sir-2.1* acts through *daf-16* to modulate lifespan, *sir-2.1* is likely to act either upstream of *age-1* or in a pathway that is parallel to the canonical *daf-2*/*age-1*/*akt-1* signaling cascade, but converges onto *daf-16*.

Considering that SPT-4, CEH-18, and DAF-16 are all transcription factors, it is possible that they function as a protein complex that regulates gene expression and longevity. However, our genetic interaction results argue against this possibility. RNAi inactivation of the transcriptional elongation factor *spt-4* or the POU-domain transcription factor *ceh-18* extends *daf-16* null mutant lifespan, suggesting that SPT-4 and CEH-18 likely regulate lifespan by functioning independent of DAF-16. RNAi of *ceh-18* requires *sir-2.1* activity to extend lifespan, suggesting that *ceh-18* might function upstream of *sir-2.1*, or cooperate with *sir-2.1* to regulate lifespan in a *daf-16*-independent manner.

Several longevity genes depend on both *daf-16* and *sir-2.1* activities to regulate lifespan. These include a WD domain protein (F21H12.1), a scramblase (K08D10.7), a copine protein (C01F6.1), a cysteine protease (Y71H2AR.2), a protein related to male fertility (C36H8.1), and two unknown proteins (R05A10.5 and Y75B8A.13) (Table 2). These genes might act upstream of both *daf-16* and *sir-2.1*, or they might cooperate with both *daf-16* and *sir-2.1* to affect lifespan. A noticeable finding from our genetic interaction analyses is that a substantial number of the candidate longevity genes require *daf-16* activity to regulate lifespan. This includes a number of proteins with unknown function, as well as proteins predicted to participate in signal transduction. Thus, in addition to *age-1* and *akt-1*, a large number of factors might function through *daf-16* to regulate lifespan. This finding is consistent with the emerging view that DAF-16 acts as a central integrator of multiple different longevity signals.

Of all the longevity regulatory genes identified by the RNAi screen, *age-1* or *akt-1* RNAi induces the most robust lifespan extension phenotype (Table 2; Supplementary Table 1). The *daf-2/age-1* insulin-like signaling pathway was the first signaling pathway identified to dramatically regulate *C. elegans* lifespan through forward genetic screens. It is interesting that this pathway stands out in two different screening strategies. It is likely that insulin-like regulation of *C. elegans* lifespan is so potent because inactivation of insulin signaling mimics a physiological regulatory event; in the dauer animal, insulin signaling is physiologically decreased (Riddle and Albert 1997) and a cascade of gene regulatory responses, including protection from free radicals and a change in metabolic rate ensues (Vanfleteren and De Vreese 1995; Honda and Honda 1999; Van Voorhies and Ward 1999; Murphy et al. 2003). Because inactivation of insulin-signaling pathway components induces a change in lifespan that has probably been under genetic selection for long-lived and stress-resistant dauer stage animals, it is not surprising that it is a robust regulator of *C. elegans* longevity.

Our screen also identified a large number of new candidate longevity genes that are predicted to participate in diverse biological functions. Although RNAi inactivation of many of these candidate genes did not produce a phenotype as robust as that of *age-1* or *akt-1* RNAi, their weaker phenotype might be due to insufficient gene knockdown, redundant function, or the need for precise gene knockdown in specific cells and/or developmental times. Some of these candidate longevity genes belong to functional classes that have not been implicated in longevity control before. Characterization of these candidate longevity genes will provide novel insights into how lifespan might be regulated in *C. elegans*.

Materials and methods

Strains

We used the following *C. elegans* strains: wild-type N2 Bristol, *rrf-3(pk1426)*, *daf-16(mgDf47);rrf-3(pk1426)*, *sir-2.1(ok434);rrf-3(pk1426)*, and *hsp-6::gfp(zcIs13)* (a kind gift from David Ron, Skirball Institute, New York).

Genome-wide RNAi lifespan screen

We carried out a large-scale RNAi screen using wild-type N2 strain as described previously (Lee et al. 2003b). Briefly, each RNAi colony was grown overnight in LB with 50 μg/mL of ampicillin and then seeded onto 24-well RNAi agar plates containing 5 mM isoprophylthiogalactoside (IPTG). The RNAi bacteria were induced overnight at room temperature for dsRNA expression. We then added ~20–40 synchronized larval stage-one animals to each well, allowed worms to develop to adults, and then added 5-fluorodexoyuridine (FUDR) solution to a final concentration of 0.1 mg/mL. Worms were kept at 25°C and their lifespan was monitored. Worms feeding on bacteria carrying the empty vector (L4440) were used as control. Worms feeding on RNAi bacteria expressing dsRNA targeting the *daf-2* gene was used as control (*daf-2* RNAi construct kindly provided by M. Vidal, Harvard Medical School, Boston, MA). We routinely screened ~500–1000 RNAi clones in one experiment. For each screening experiment, at least 48 wells of L4440 control were included. We monitored the control worm populations regularly. At the time when all of the control worms were dead, we scored each well containing the different RNAi bacteria for possible live worms. RNAi wells in which live worms were observed were scored as positives. These "positive" RNAi clones were retested at least two more times using similar high-throughput screening strategy described above.

RNAi clones that were scored as positive in rescreening were deemed "primary positives". Primary positives were retested two or three times in conventional RNAi lifespan assays (see below) using the *rrf-3(pk1426)* strain. Because each of the final 90 RNAi clones were scored positive at least twice in our initial high-throughput screening using N2 worms, and were subsequently scored positive at least two more times using *rrf-3(pk1426)* worms in conventional lifespan assays, we are confident that reduced function of each of these genes contributes to lifespan extension. Because RNAi often only partially inactivates a gene function, it is very possible that a more severe attenuation of some of these genes would extend lifespan more. This is, in fact, observed with our positive control *daf-2* RNAi. The control *daf-2* RNAi construct we used (kindly provided by M. Vidal) induces ~50% lifespan increase, and the *daf-2* RNAi construct from the genome-wide library only induces ~20% lifespan increase. In contrast, a temperature-sensitive genetic mutation of *daf-2* extends lifespan up to threefold (Kenyon et al. 1993; Morris et al. 1996; Kimura et al. 1997; Paradis and Ruvkun 1998; Paradis et al. 1999). Some of the RNAi clones induced lifespan extension in only two of the three retests. Variability of RNAi efficacy among experiments has been observed previously (Simmer et al. 2003).

RNAi lifespan assay

RNAi bacteria were prepared as described above. Gravid *rrf-3(pk1426)* worms were allowed to lay eggs onto RNAi bacteria plates containing 2 mM IPTG at 15°C for ~12 h. The progeny were allowed to grow to adults at 25°C. At 25°C, a large proportion of the *rrf-3(pk1426)* worms became sterile adults. Approximately 40–60 of the adult animals undergoing RNAi treatment were then transferred to RNAi agar plates containing 0.05–0.1 mg/mL of FUDR that were seeded with freshly induced RNAi bacteria of interest. The animals were then kept at either 25°C or 20°C, and scored every 2 d by gentle prodding with a platinum wire to test for live or dead. To ensure the continued

efficacy of RNAi knockdown, animals were fed freshly induced RNAi bacteria every 5 d. Lifespan is defined as the time elapsed from when worms were put on FUDR plates (adult lifespan = 0) to when they were scored as dead. Worms that died of protruding/bursting vulva, bagging, or crawling off the agar were censored.

For epistasis analysis, RNAi lifespan assays were performed as described above, except either *daf-16(mgDf47);rrf-3(pk1426)* or *sir-2.1(ok434);rrf-3(pk1426)* worms were used.

Statistical analysis

Statistical analyses were performed using the software SPSS (SPSS Inc.). The survival experience of each RNAi-treated population is compared with that of the population treated with control RNAi using the log rank test. A P-value < 0.05 was considered as significantly different from control.

hsp-6::gfp *assay*

Transgenic animals carrying an integrated transgene of *hsp-6::gfp* (Yoneda et al. 2004) were allowed to lay eggs onto RNAi bacteria as described above. The fluorescence intensity of each population was monitored as soon as worms reached young adulthood. Images of each population of RNAi-treated transgenic animals were captured using the OpenLab software. All of the images were taken with the same amount of exposure time.

Informatics

Analysis of how the candidate longevity genes fit into the TOPO expression mountains was performed via http://workhorse.stanford.edu/cgi-bin/gl/gene_list.cgi?set=2 (see also Kim et al. 2001). Analysis of possible protein–protein interaction among the candidate longevity genes was performed via the Interactome query page (http://vidal.dfci.harvard.edu).

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