tion. The Mvp1p-homology domain of SNX1 is a candidate for interacting with other components of the vesicular trafficking machinery to segregate EGFR into endosomal membranes targeted for degradation. These features define a family of proteins that confer specificity on receptor sorting in the endocytic pathway. As a negative regulator of EGFR on cell surfaces, SNX1 influences the efficiency with which EGF activates intracellular signal transduction pathways.

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5. The two-hybrid screening system consisted of yeast strain EGY48 (*MAT***a** *trp1 ura3 his3 LEU2::pLexAop6-LEU2*); plasmids pEG202, pJG4-5, pSH18-34, and pRFHM1; and a HeLa library in pJG4-5 (*14*). A cDNA encoding EGFR residues 663 to 958 was obtained by polymerase chain reaction (PCR) with Pfu polymerase (Stratagene, La Jolla, CA) by using a human EGFR cDNA and cloned into the yeast LexA fusion expression plasmid pEG202. Interactor clones were selected by leucine auxotrophy and galactose-inducible β-galactosidase activity. Library plasmids were recovered from positive colonies with *Escherichia coli* strain KC8 (*pyrF::*Tn*5, hsdr, leuB600, trpC9830, lacD74, strA, galK, hisB436*) and retransformed into EGY48 yeast carrying plasmid (pRFHM1) containing a LexA-bicoid fusion (*4*) to eliminate nonspecific binders. The 5′ ends of specific plasmids were sequenced by cycle sequencing (Epicentre Technologies, Madison, WI) with ^{35}S-labeled deoxyadenosine triphosphate and a primer (5′-GATGTTAACGATACCAGCCTCTTGCTGAGT-3′) that bound upstream of the vector-cDNA fusion junction. The sequence was used to search the nucleotide sequence databases of the National Center for Biotechnology Information using the BLAST algorithm (*15*).
6. A HeLa λgt11 cDNA library (Clontech, Palo Alto, CA) was plated and screened with a ^{32}P-labeled oligonucleotide complementary to the 5′ end of the SNX1 library plasmid insert (5′-CTTTCTCAAACCTCACTTCT-3′). Six phages were plaque-purified, the cDNAs subcloned into the Eco RI site of pMOBII (Gold Biotechnology, St. Louis, MO), and transposon insertions used to determine the complete sequence of two of the inserts on both strands. Areas of ambiguity were clarified with gene-specific primers. The sequence of SNX1 is deposited in GenBank (accession number U53225).
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12. An SNX1 (residues 1 to 522) expression vector was prepared in pCEP4 (Invitrogen, San Diego, CA). Transfections were performed by exposure of cells on 6-cm-diameter plates to calcium phosphate DNA coprecipitates (15 μg per 2 × 10^6 cells) for 4 to 6 hours. For transient assays, lysates from human embryonic kidney 293 cells were prepared 48 hours later for protein immunoblotting. Stable African green monkey kidney CV-1 cells were prepared by expanding the cells 48 hours after transfection onto 10-cm-diameter plates in the presence of hygromycin (320 μg/ml) and selected for 14 to 21 days. Clonal lines were prepared from isolated colonies with cloning rings.
13. For the determination of EGFR half-life, CV-1 cells were grown in the absence of methionine in Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum for 2 hours and incubated with ^{35}S-methionine (0.65 mCi/ml) for 1 hour. Cells were incubated in complete medium, and radio immunoprecipitation assay extracts [1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM Hepes (pH 7.6), and 150 mM NaCl] were prepared after incubation for various times and immunoprecipitated with antibodies 528 and 13A9 to EGFR. After they were washed, the immunoprecipitates were solubilized in SDS sample buffer, electrophoresed, and visualized by fluorography, and the radioactivity was quantified by liquid scintillation counting.
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16. The detergent extraction buffer was 1% Triton X-100, 50 mM Hepes (pH 7.4), 10% glycerol, and 75 mM NaCl. Whole-cell lysate buffer was 1% SDS and 10 mM Hepes (pH 7.4). Buffers were supplemented with the following phosphatase and protease inhibitors: 10 mM NaF, 1 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, leupeptin (10 μg/ml), antipain (10 μg/ml), and aprotinin (10 μg/ml). Products of the coupled rabbit reticulocyte lysate transcription and translation reaction (Promega, Madison, WI) were separated by gel electrophoresis and visualized by fluorography.
17. Cells were fixed in paraformaldehyde; permeabilized with saponin; stained with antibody to SNX1 (anti-SNX1) and a mixture of antibodies to EGFR [immunoglobulin G's (IgGs) 528, 13A9, and 225], followed by Texas Red–conjugated goat anti-rabbit IgG and fluorescein isothiocynate–conjugated goat anti-mouse IgG; and visualized by epifluorescence illumination.
18. We generated the cDNAs encoding EGFR residues 647 to 1186, 647 to 957, and 647 to 942 by PCR with Pfu polymerase (Stratagene) using a human EGFR cDNA, and the products were cloned into the yeast LexA fusion expression plasmid pEG202. The rat ERBB2 core tyrosine kinase domain was generated by PCR. For β-galactosidase assay, extracts were prepared from logarithmic-phase yeast diluted into Ura$^-$His$^-$Trp$^-$ broth containing 2% dextrose or 2% galactose (to induce the library plasmid insert) and grown at 30°C for 18 to 22 hours (*14*).

19. We thank R. Brent for providing the components of the yeast two-hybrid system and A. Nesterov and H. S. Wiley for helpful discussions. Supported by NIH grants F32DK08666 (to R.C.K.) and CA58689 (to G.N.G.) and by funds provided by the Breast Cancer Fund of the State of California through the Breast Cancer Research Program of the University of California, grant numbers 1FB-0314 (to R.C.K.) and 1KB-0140 (to D.L.C.).

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Determination of Life-Span in *Caenorhabditis elegans* by Four Clock Genes

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The nematode worm *Caenorhabditis elegans* is a model system for the study of the genetic basis of aging. Maternal-effect mutations in four genes—*clk-1, clk-2, clk-3,* and *gro-1*—interact genetically to determine both the duration of development and life-span. Analysis of the phenotypes of these mutants suggests the existence of a general physiological clock in the worm. Mutations in certain genes involved in dauer formation (an alternative larval stage induced by adverse conditions in which development is arrested) can also extend life-span, but the life extension of Clock mutants appears to be independent of these genes. The *daf-2(e1370) clk-1(e2519)* worms, which carry life-span–extending mutations from two different pathways, live nearly five times as long as wild-type worms.

It is not known why organisms senesce and die. One set of theories suggests that life-span is timed, much like puberty and menopause (*1*). Another set suggests that organisms accumulate damage throughout life, which eventually leads to the failure of one or more critical physiological systems (*2, 3*). One of the latter theories, which has some experimental support, posits that senescence may result from oxidative damage caused by the reactive by-products of metabolism (*3, 4*). One way to investigate the nature of aging is to study long-lived mutants. Here we describe four genes that interact genetically to determine both the duration of postembryonic development and the adult life-span of the nematode worm *Caenorhabditis elegans*.

In a screen for maternally rescued viable mutations in *C. elegans*, we recovered mutations in three genes—*clk-1*, *clk-2*, and *clk-3*—that show the Clock (Clk) phenotype, a pleiotropic alteration of developmental and behavioral timing (*5, 6*). The phenotype of *gro-1(e2400)* places it, too, in the Clk class of genes (*5*). Mutations in the best characterized of these genes, *clk-1*, lengthen early embryonic cell cycles, embryonic and postembryonic development, as well as the period of rhythmic adult behaviors, such as swimming, pharyngeal pumping, and defecation (*5*). Furthermore, *clk-1* mutants have a longer mean and maximum life-span than the standard wild-type strain N2 (*5*).

To characterize these genes further, we determined the life-span of the reference strain of these genes at 15°, 18°, 20°, and 25°C. All four mutants have longer mean and maximum life-spans than the wild type at all temperatures (Table 1). For example, at 18°C, all Clk mutants have a mean life-span at least 3 days longer than that of the

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Table 1. Mean life-span of Clk strains at 15°, 18°, 20°, and 25°C. The aging study was done as described (*5*), except that eggs were allowed to hatch for up to 6 hours (for N2 and the single mutants) or up to 32 hours (for Clk double mutants). Only reference alleles were used, except for *fer-15(b26)* and *clk-1(qm30)*. Strains were constructed by standard techniques (*17*). At each temperature, the mean life-span and standard error are given. The numbers given in parentheses are the number of separate experiments in which the life-span of a cohort (usually 50 worms) was examined and the total pooled sample size, respectively. Repeated experiments gave very similar results. ND, not determined. Strains carrying *gro-1*, *clk-2*, or *fer-15* mutations are not fertile at 25°C, so in the experiments at this temperature, worms were allowed to hatch at 20°C and then were transferred to 25°C.

Genotype	Mean life-span (days) at temperature			
	15°C	18°C	20°C	25°C
N2	22.0 ± 0.3 (5, 236)	14.9 ± 0.3 (5, 250)	16.1 ± 0.2 (5, 242)	9.2 ± 0.3 (2, 100)
clk-1	29.3 ± 0.5 (5, 212)	18.4 ± 0.4 (5, 250)	17.3 ± 0.4 (5, 239)	11.6 ± 0.5 (2, 87)
*clk-1**	31.0 ± 0.9 (2, 100)	19.8 ± 0.5 (3, 149)	19.3 ± 0.6 (3, 147)	11.1 ± 0.2 (1, 43)
clk-2	24.6 ± 0.8 (3, 139)	18.7 ± 0.5 (3, 150)	18.0 ± 0.5 (4, 191)	11.7 ± 0.7 (1, 50)
clk-3	25.7 ± 0.7 (2, 100)	20.4 ± 0.4 (5, 250)	19.9 ± 0.6 (3, 147)	13.0 ± 0.8 (1, 50)
gro-1	26.0 ± 0.7 (3, 125)	19.2 ± 0.6 (4, 200)	19.7 ± 0.6 (3, 147)	15.6 ± 0.5 (1, 50)
clk-1 clk-2	34.5 ± 0.9 (2, 100)	28.2 ± 0.9 (3, 136)	23.1 ± 0.7 (3, 143)	ND
*clk-1 clk-2**	30.3 ± 0.9 (1, 50)	28.3 ± 0.7 (1, 50)	25.4 ± 0.7 (1, 50)	ND
clk-3; clk-1	36.7 ± 1.4 (2, 69)	22.7 ± 0.6 (3, 99)	17.8 ± 0.5 (3, 138)	17.4 ± 1.4 (1, 10)
*clk-3; clk-1**	41.4 ± 1.7 (2, 79)	43.5 ± 1.5 (1, 50)	27.5 ± 1.2 (2, 105)	ND
clk-3; clk-2	33.8 ± 0.9 (2, 100)	22.3 ± 0.8 (3, 150)	20.6 ± 0.6 (3, 144)	12.7 ± 0.5 (1, 50)
gro-1 clk-2	21.4 ± 0.9 (1, 37)	12.3 ± 0.6 (1, 50)	ND	ND
clk-3; gro-1	24.4 ± 0.6 (1, 50)	15.9 ± 0.8 (2, 82)	14.6 ± 0.4 (3, 145)	ND
age-1 fer-15	30.9 ± 1.2 (2, 100)	26.3 ± 1.2 (2, 94)	26.6 ± 0.8 (3, 149)	22.0 ± 1.5 (1, 50)
age-1 fer-15; gro-1	ND	34.1 ± 1.7 (1, 50)	ND	30.6 ± 0.9 (1, 50)

*These strains contain *clk-1(qm30)*. All other *clk-1* strains contain *clk-1(e2519)*.

wild type (Table 1 and Fig. 1). The extended life-span of *gro-1* mutants has been noted (*7*).

To examine how these genes interact genetically, we made all but one of the possible double mutant combinations containing the reference allele of each gene (*8*). Although there is a range of severity, all double mutants take longer to develop than those with mutations in the individual constituent genes (Table 2). We examined the life-spans of double mutants at 15°, 18°, and 20°C, and in selected cases at 25°C as well. Most double mutants have much longer mean and maximum life-spans than the individual Clk mutants, especially at low temperatures (Table 1). For example, the mean life-span of *clk-1(e2519) clk-2(qm37)* mutants is more than 50% greater than that of either *clk-1(e2519)* or *clk-2(qm37)* alone and almost 90% greater than that of the wild type at 18°C (Fig. 1A and Table 1).

To determine the specificity of the interactions among the Clk genes, we made two sets of *clk-1 clk-2* and *clk-3*; *clk-1* double mutants, one containing *clk-1(e2519)* and the other *clk-1(qm30)*. The *clk-1* allele *qm30* is much stronger than *e2519* for almost all phenotypes examined except for its effect on life-span, for which the two alleles do not appear to be different [Table 1 and (*5*)]. The *clk-3(qm38); clk-1(qm30)* double mutants have a very long mean life-span and development at all temperatures, up to three and four times that of the wild type,

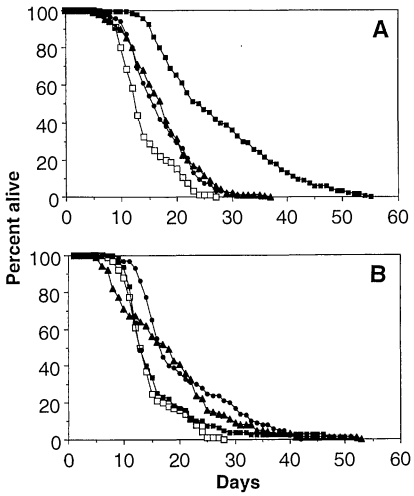

Fig. 1. Clock mutations interact to determine life-span. Graphs show the percentage of worms alive on a given day after hatching (day 0) at 18°C. (**A**) N2 (□), *clk-1(e2519)* (●), *clk-2 (qm37)* (▲), and *clk-1(e2519) clk-2(qm37)* (■). Sample size (*n*) is 150, except for *clk-1 clk-2* double mutants (*n* = 136). The *clk-1 clk-2* double mutants live even longer than *clk-1* and *clk-2* single mutants. (**B**) N2 (□), *clk-3* (●), *gro-1* (▲), and *clk-3; gro-1* (■); *n* = 100 except for *clk-3; gro-1* double mutants (*n* = 82). The *clk-3; gro-1* double mutants have a mean life-span similar to that of the wild type, even though each mutation individually increases life-span.

Table 2. Length of postembryonic development of Clk strains at 15°, 18°, 20°, and 25°C. Eggs were allowed to hatch for up to 12 hours, and 25 worms were then scored for their developmental stage every 12 hours until all became adults. The time noted is the length of time between the midpoint of the hatching window to the midpoint of the window in which the median worm became an adult. The stated error is the sum of the length of the hatching window and the adulthood window divided by 2. ND, not determined.

Genotype	Development (days) at temperature			
	15°C	18°C	20°C	25°C
N2	4.1 ± 0.4	2.5 ± 0.3	2.4 ± 0.3	1.5 ± 0.5
clk-1	5.0 ± 0.4	3.0 ± 0.3	2.9 ± 0.3	2.5 ± 0.4
*clk-1**	6.6 ± 0.4	4.0 ± 0.3	3.9 ± 0.3	2.5 ± 0.4
clk-2	5.1 ± 0.4	3.0 ± 0.3	2.9 ± 0.3	4.3 ± 0.4
clk-3	5.0 ± 0.4	3.5 ± 0.3	2.9 ± 0.3	2.4 ± 0.4
gro-1	7.6 ± 0.4	4.1 ± 0.4	3.4 ± 0.3	2.9 ± 0.3
clk-1 clk-2	10.0 ± 0.4	6.0 ± 0.4	6.5 ± 0.5	ND
*clk-1 clk-2**	9.0 ± 0.4	6.5 ± 0.4	7.0 ± 0.5	ND
clk-3; clk-1	7.1 ± 0.4	5.8 ± 0.3	4.1 ± 0.4	4.5 ± 0.5
*clk-3; clk-1**†	11.4 ± 0.5	10.5 ± 0.5	12.0 ± 0.4	ND
clk-3; clk-2	5.5 ± 0.5	3.9 ± 0.3	3.8 ± 0.3	5.5 ± 0.5
gro-1 clk-2	8.9 ± 0.4	4.4 ± 0.3	ND	ND
clk-3; gro-1	10.0 ± 0.4	8.0 ± 0.4	5.5 ± 0.5	ND
age-1 fer-15	4.3 ± 0.3	3.0 ± 0.3	2.4 ± 0.3	1.5 ± 0.5
age-1 fer-15; gro-1	ND	3.6 ± 0.4	ND	3.3 ± 0.3

*These strains contain *clk-1(qm30)*. All other *clk-1* strains contain *clk-1(e2519)*. †It was difficult to accurately determine when *clk-3(qm38); clk-1(qm30)* worms completed development, especially at 20°C, because these worms never develop a functional germ line. The number given is a maximum estimate.

respectively, and much longer than that of *clk-3(qm38); clk-1(e2519)* double mutants (Tables 1 and 2). In contrast to *clk-3; clk-1* double mutants, *clk-1 clk-2* double mutants with either *clk-1(qm30)* or *clk-1(e2519)* develop at the same rate and have equally extended life-spans (Tables 1 and 2), which suggests that there is a high degree of specificity in the interaction of *clk-1* and *clk-3*.

The interactions of *gro-1* with *clk-2* and *clk-3* are distinct from those between other pairs of Clk mutants. Although *clk-2*, *clk-3*, and *gro-1* single mutants all live longer than the wild type (Table 1), both *gro-1 clk-2* and *clk-3; gro-1* double mutants have a mean life-span similar to that of the wild type (Fig. 1B and Table 1). However, *gro-1* does not suppress the increased longevity of *age-1(hx546)*, another mutation that confers long life (9). In fact, the mean life-span of an *age-1 fer-15; gro-1* strain is greater than that of *gro-1* and *age-1 fer-15* strains [Table 1 and (*10*)]. These results suggest that the suppression of extended life-span of *clk-2* and *clk-3* by *gro-1* involves a specific interaction between these genes and shows that *gro-1* and the *clk* genes function in a common pathway.

Several other *C. elegans* genes are known to affect life-span (*9*, *11*–*13*). The best-studied of these are involved in the control of dauer formation [where dauer refers to a nonfeeding, alternative third larval stage that is induced by adverse conditions and can live for up to 6 months (*14*)]. Certain mutations can cause either constitutive (Daf-c) or defective (Daf-d) dauer formation (*14*). Mutations in the Daf-c genes *daf-2* and *daf-23* can lead to an extended life-span without entry into the dauer stage (*12*, *13*). Mutations in the Daf-d gene *daf-16* suppress extended life-span (as well as the Daf-c phenotype) in *daf-16; daf-2* and *daf-16; daf-23* double mutants (*12*, *13*). *daf-16* also suppresses the increased longevity of *age-1* mutants, linking longevity in *age-1* mutants to the dauer pathway (*13*). The effect of Clk genes on life-span, however, appears to involve a *daf-16*–independent mechanism: *daf-16(m26)* does not suppress the life-span extension seen in *clk-1*, *clk-3*, or *gro-1* strains (Fig. 2A and Table 3); we did not test its effects on *clk-2*.

Because the dauer genes and the Clk genes appear to affect life-span by different mechanisms, we examined the effect on life-span when the worms carried mutations in both a Daf-c and a Clk gene. A *daf-2(e1370) clk-1(e2519)* strain lives longer than its component strains at both 18° and 25°C (Table 3 and Fig. 2B). Furthermore, even though the adult life-span of *clk-1* mutants at 25°C is not different from that of the wild type, the mean life-span and mean adult life-span of *daf-2 clk-1* double mutants is nearly five and six times those of the wild type, respectively (Table 3). This is the largest increase in mean life-span over the species average seen in any organism (*12*, *13*).

The lengthened life-span of Clk mutants is not due solely to lengthened development. Clk mutants and most double mutants have significantly longer mean adult life-spans than the wild type. We plotted the length of development against mean adult life-span for all experiments done at a constant temperature (Fig. 3). Lowering temperature lengthens both development and mean adult life-span in the wild type in an apparently linear fashion [Fig. 3 and (*15*)]. Most other strains, including the Clk single mutants and all *clk* double mutants, display a similar relation between these two life history traits. However, at any given temperature, these strains have both a long-

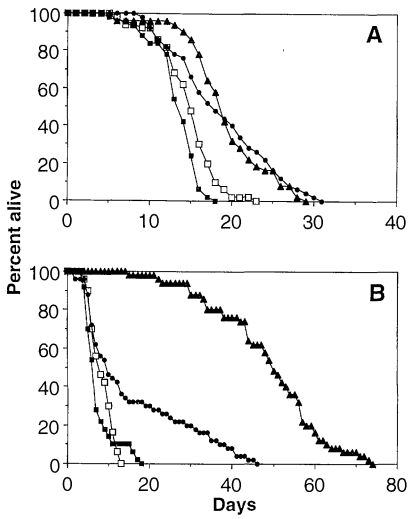

Fig. 2. Interaction of *clk-1* with *daf-2* and *daf-16*. (**A**) Percentage of worms alive on a given day after hatching (day 0) at 18°C: N2 (□), *daf-16(m26)* (■), *clk-1(e2519)* (●), and *daf-16(m26); clk-1(e2519)* double mutants (▲). Sample size (*n*) is 50 for all genotypes. *daf-16; clk-1* double mutants are indistinguishable from *clk-1* mutants, indicating that *daf-16* does not suppress longevity in *clk-1* mutants. (**B**) Percentage of worms alive on a given day after their first day of adulthood (day 0) at 25°C: N2 (□), *clk-1(e2519)* (■), *daf-2(e1370)* (●), and *daf-2(e1370) clk-1(e2519)* (▲); *n* = 50 for all genotypes. Worms were raised at 20°C until the first day all worms were adults to prevent *daf-2(e1370)* mutants from becoming dauers (*12*) and then were transferred to 25°C. N2 and *daf-2* were transferred after 3 days, *clk-1* after 4 days, and *daf-2 clk-1* after 6 days. The adult life-span of *daf-2 clk-1* double mutants is considerably longer than that of *daf-2* alone and almost six times that of the wild type.

Table 3. The interaction of Clk genes with Daf genes. Mean life-span and development were scored as in Tables 1 and 2. Double mutants were constructed as described (*17*). Some controls for experiments at 15° and 18°C are given in Tables 1 and 2.

Genotype	Temperature (°C)	Mean life-span (days)	Development (days)
daf-16	18	15.1 ± 0.3 (2, 150)	2.5 ± 0.3
daf-16; clk-1	18	20.3 ± 0.7 (1, 50)	3.4 ± 0.3
daf-16; clk-3	18	19.2 ± 0.8 (1, 50)	3.9 ± 0.3
daf-16; gro-1	18	17.8 ± 0.6 (1, 50)	3.9 ± 0.3
daf-2; clk-1	15	43.3 ± 1.5 (1, 50)	6.0 ± 0.5
daf-2	18	29.7 ± 1.4 (2, 100)	3.0 ± 0.3
daf-2; clk-1	18	34.0 ± 1.8 (2, 100)	4.0 ± 0.3
*N2**	25	8.5 ± 0.4 (1, 50)	2.4 ± 0.3
*clk-1**	25	8.6 ± 0.5 (1, 50)	2.9 ± 0.3
*daf-2**	25	15.9 ± 1.8 (1, 50)	2.9 ± 0.3
*daf-2; clk-1**	25	49.1 ± 1.9 (1, 50)	4.3 ± 0.3

*These experiments were done under the conditions described in Fig. 2B; only adult life-span is given and development was scored at 20°C.

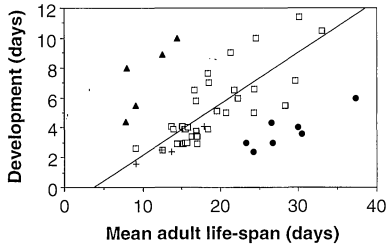

Fig. 3. Scatter plot of the length of development versus mean adult life-span. Data are shown for all experiments in which strains were maintained at a constant temperature. Mean adult life-span was calculated (mean life-span — length of development) with the use of the data in Tables 1 through 3. Symbols are as follows: +, N2; ●, *daf-2*– and *age-1*–containing strains; ▲, *gro-1 clk-2* and *clk-3; gro-1* double mutants; □, all other strains, including Clk single mutants and all Clk double mutants that do not contain *gro-1(e2400)*. A linear regression line is shown for the last group of strains. Also included in this plot are points for N2 [life-span = 10.7 ± 0.3 days (1, 200), development = 1.6 ± 0.4 days] and *clk-1(e2519)* [life-span = 11.7 ± 0.3 days (1, 200), development = 2.6 ± 0.4 days] continuously cultured at 25°C (numbers in parentheses are according to the guidelines listed in Table 1). Because of the difficulty in determining the length of development of *clk-3(qm38); clk-1(qm30)* worms at 20°C (see Table 2), these results were excluded.

er development and longer mean adult life-span than the wild type. It appears therefore that mutations in the Clk genes have similar effects on development and on life-span as has lowered temperature.

Two groups of strains have a very distinct relation between adult life-span and the length of development: one group includes *gro-1 clk-2* and *clk-3*; *gro-1* double mutants and the second includes all strains containing either *daf-2* or *age-1*. At any given temperature, *gro-1 clk-2* and *clk-3*; *gro-1* double mutants have a much longer development and a shorter adult life-span than the wild type (Fig. 3). In contrast, all strains containing either *daf-2* or *age-1* have a much longer mean adult life-span, relative to development, than the wild type (Fig. 3). The *daf-2 clk-1* and *age-1 fer-15*; *gro-1* strains, which are part of the second group, display the effects of both of the life-extending mutations they contain. These strains show the typical lengthening of adult life-span due to *daf-2* or *age-1*, and in addition, *clk-1* or *gro-1* lengthen their development and increase their life-spans even further.

We propose that the Clk genes are involved in the general control of timing in *C. elegans*, perhaps constituting parts of a general physiological clock. Because, in Clk mutants, life appears to proceed at a slower pace, our results are consistent with the "rate of living" theory of aging (*1*, *16*). Conceivably, the slower rate of living of Clk mutants could be accompanied by a lowered rate of metabolism. However, the possibly slower metabolic rates of Clk mutants are probably not due to a defect in a key metabolic enzyme, but rather due to an altered control of metabolism. All Clk mutations are fully maternally rescued for almost all timing defects (*5*, *6*). This means that homozygous mutant progeny of a heterozygous mother have a nearly wild-type phenotype. It is unlikely that a gene coding for a key metabolic enzyme, required in even moderate amounts in every cell throughout life, could display this form of inheritance. This result, along with certain other aspects of the *clk-1* phenotype (*5*), suggests that the Clk genes are probably regulatory genes that may, among other things, control metabolic rate. Although how the slower rate of living seen in Clk mutants leads to a longer life-span is unknown, a reduced metabolic rate could lead to a slower production of reactive by-products of metabolism and, thus, to more gradual aging.

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17. All mutations used in this study, except *age-1(hx546)*, have scorable phenotypes distinct from their effects on life-span. These were used in the construction of multiple mutant strains. All genes used in the aging study are contained on LG III except *age-1*, *fer-15*, *clk-3* (LG II), and *daf-16* (LG I). We constructed strains containing unlinked mutations by generating heterozygotes with one of the mutations balanced over closely linked markers in trans, making the unbalanced mutation homozygous (as scored by its phenotype) and then making the second mutation homozygous by displacement of trans markers. Linked doubles between LG III mutations were constructed with the use of appropriate flanking markers to pick recombinants and then with the removal of those flanking markers by outcrossing. All double mutants constructed had developmental phenotypes fully consistent with their presumed genotypes. In addition, the presence of *daf-16(m26)* in the putative *daf-16* double mutants was confirmed by a complementation test in which the ability to suppress *daf-2(e1370)* was scored. The *age-1 fer-15; gro-1* strain develops slowly and is sterile at 25°C, confirming the presence of *gro-1* and *fer-15*, respectively. Because this strain lives considerably longer than *gro-1* and because *fer-15* has no known effect on life-span (*9*), we conclude that *age-1* is also present in this strain.

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The Role of Zinc in Selective Neuronal Death After Transient Global Cerebral Ischemia

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Zinc is present in presynaptic nerve terminals throughout the mammalian central nervous system and likely serves as an endogenous signaling substance. However, excessive exposure to extracellular zinc can damage central neurons. After transient forebrain ischemia in rats, chelatable zinc accumulated specifically in degenerating neurons in the hippocampal hilus and CA1, as well as in the cerebral cortex, thalamus, striatum, and amygdala. This accumulation preceded neurodegeneration, which could be prevented by the intraventricular injection of a zinc chelating agent. The toxic influx of zinc may be a key mechanism underlying selective neuronal death after transient global ischemic insults.

Chelatable zinc (Zn^{2+}) is present in presynaptic vesicles of central excitatory neurons (*1*) and is released with synaptic activity or membrane depolarization (*2*). Although the precise role of synaptically released Zn^{2+} is not known, Zn^{2+} blocks currents mediated by *N*-methyl-D-aspartate (NMDA) (*3*, *4*) and by γ-aminobutyric acid (GABA) (*3*) as well as voltage-gated calcium channels (*5*). In addition, exposure to excessive extracellular Zn^{2+} is neurotoxic to cortical neurons; this toxicity may be mediated in part by Zn^{2+} influx through glutamate receptor- or voltage-gated Ca^{2+} channels (*6*). Presynaptic Zn^{2+} translocates into selectively vulnerable hippocampal hi-

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