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Extension of Life-Span by Loss of CHICO, a *Drosophila* Insulin Receptor Substrate Protein

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The *Drosophila melanogaster* gene *chico* encodes an insulin receptor substrate that functions in an insulin/insulin-like growth factor (IGF) signaling pathway. In the nematode *Caenorhabditis elegans*, insulin/IGF signaling regulates adult longevity. We found that mutation of *chico* extends fruit fly median life-span by up to 48% in homozygotes and 36% in heterozygotes. Extension of life-span was not a result of impaired oogenesis in *chico* females, nor was it consistently correlated with increased stress resistance. The dwarf phenotype of *chico* homozygotes was also unnecessary for extension of life-span. The role of insulin/IGF signaling in regulating animal aging is therefore evolutionarily conserved.

Mutations that extend life-span illuminate the molecular mechanisms underlying aging and longevity. In *Caenorhabditis elegans*, mutation of the genes *daf-2* and *age-1*, which encode components of an insulin/IGF signaling (IIS) pathway, enhances stress resistance and increases adult life-span by up to 200% (*1*). This pathway also controls the formation of dauer larvae, which are developmentally arrested, stress resistant, long-lived, and produced in response to crowding and reduced food (*2*). Potentially, insulin/IGF mutants could be long-lived by virtue of expression of dauer longevity in the adult, in which case the extension of adult life-span by these mutations could be a peculiarity of *C. elegans*. We examined whether the role of IIS in aging has been evolutionarily conserved and therefore might also operate in humans.

In the fruit fly *Drosophila melanogaster*, the insulin/IGF receptor INR, the insulin receptor substrate CHICO, the phosphatidylinositol 3-kinase (PI3K) Dp110/p60, and the PI3K target protein kinase B (PKB, also known as DAkt1) form a signaling pathway that regulates growth and size (*3–7*). We examined the effects on aging of hypomorphic mutations in *Inr* (equivalent to *daf-2*) and *PKB*, and null mutations in *chico* and the catalytic (*Dp110*, equivalent to *age-1*) and adapter (*p60*) PI3K subunits (*8*). All mutants were tested as heterozygotes. We also tested $chico^1$ (*3*) and PKB^3 (*9*) homozygotes and Inr^{GC25}/Inr^{E19} transheterozygotes, which form viable dwarf adults. The remaining mutations were homozygous lethal.

Most mutants tested had normal or significantly decreased life-span (*10*). For example, PKB^3 homozygotes and Inr^{GC25}/Inr^{E19} flies were short-lived. By contrast, $chico^1$ extended life-span (Fig. 1). Homozygous $chico^1$ females exhibited an increase of median and maximum life-span of up to 48 and 41%, respectively. $chico^1$ heterozygotes also exhibited increases in median life-span of up to 36 and 13% in females and males, respectively. Homozygous males, however, were slightly short-lived.

To confirm that $chico^1$ itself extended life-span, we tested the effect on life-span of pCSR4-*chico*, a P element containing *chico*(+). This construct fully rescues the dwarf phenotype of $chico^1$ (*3*). $chico^1$ was crossed to two stocks containing independent pCSR4-*chico* insertions (pCSR4-*chico* 1.1 and 2.3). As a control, $chico^1$ was also crossed to the base stock in which the P element insertions were made. Progeny with either two copies ($chico^1$ heterozygotes with one *chico* transgene) or one copy ($chico^1$ heterozygotes alone) of *chico*(+) were compared (*11*). The rescue construct significantly reduced life-span relative to the $+/chico^1$ control. The median female life-span of 54 days in $+/chico^1$ was reduced to 46 days in $+/chico^1$, +/*pCSR4-chico* 1.1 flies and 52 days in $+/chico^1$, +/*pCSR4-chico* 2.3 flies ($P = 0.0002$ and 0.0243, respectively). Similar effects were observed in males (*10*). Thus, mutation of *chico* itself increases life-span. Because $chico^1$ is a null allele, its effect on life-span indicates that the wild-type *chico* gene acts to accelerate aging.

Of the mutations tested, only $chico^1$ increased life-span. This may be because the effect of reduced IIS on life-span depends on the degree to which signaling is reduced. Unlike the other null mutations in IIS genes tested, $chico^1$ is not homozygous lethal, presumably because the INR receptor can signal to PI3K directly, as well as indirectly via CHICO (*3*). Thus, $chico^1$ mutants may be long-lived because of the relatively mild reduction in pathway activity that they bring about. Notably, severe IIS mutations in *C. elegans* can cause premature mortality in some adults, although the maximum life-span of populations is invariably increased (*1*). This is probably why Inr^{GC25}/Inr^{E19} flies are short-lived: Demographic analysis indicates that a reduction in the age-specific mortality rate acceleration occurs, whose effect on survival is masked by an elevated rate of age-independent mortality (*12*). Furthermore, a different heteroallelic *Drosophila Inr* mutant to that tested here exhibits an 85% increase in female life-span (*13*). By contrast, in short-lived PKB^3 populations, no reduction in mortality rate acceleration is seen (*12*). This raises the possibility that a second pathway downstream of *chico* might regulate aging in *Drosophila*. Interestingly, CHICO contains potential binding sites for the Drk/Grb2 docking protein, consistent with signaling via Ras/mitogen-activated protein kinase.

We next investigated whether extension of life-span by $chico^1$ was mediated by processes previously shown to affect aging. A reduction in fecundity extends life-span in *Drosophila* females (*14, 15*); $chico^1$ heterozygous females have reduced fecundity, and the homozygotes are almost sterile (*3, 12*). To test whether the

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increased life-span of *chico*^{1} females was due to reduced fecundity, we examined the interactions between *chico*^{1} and the dominant, female-sterile mutant *ovo*^{D1}. This mutation blocks oogenesis at stage 4, before vitellogenesis commences (*16*), and extends female life-span (*15*). If *chico*^{1} extends female life-span by the exact same mechanism as *ovo*^{D1}, then the three sterile genotypes (*chico*^{1}, +/*ovo*^{D1}, and +/*ovo*^{D1} +/*chico*^{1}) should have similar life-spans and live longer than the subfertile *chico*^{1} heterozygotes. In fact, the *chico* homozygotes lived significantly longer than all other genotypes (Fig. 2). In addition, the partially fertile *chico*^{1} heterozygotes lived as long as the sterile flies that were heterozygous for both *ovo*^{D1} and *chico*^{1} and lived significantly longer than the sterile *ovo*^{D1} heterozygotes. The effect of *chico*^{1} on female life-span is therefore not a consequence of the same mechanism of reduced fecundity as is produced by *ovo*^{D1}. If *chico*^{1} does extend female life-span through an effect on reproductive effort, the interaction must occur through some process other than oogenesis (for instance yolk protein synthesis) or before stage 4 in oogenesis because *ovo*^{D1} flies are blocked at that stage.

In *C. elegans*, long-lived IIS mutants are stress resistant and overexpress the antioxidant enzyme superoxide dismutase (SOD) (*1*). We examined the resistance of *chico*^{1} flies to three stressors, but only one showed any correspondence with life-span (*17*) (Fig. 3). No resistance to heat stress (37°C) was seen (Fig. 3A). Slight resistance to oxidative stress (methyl viologen) was observed in *chico*^{1} heterozygotes but not in homozygotes (Fig. 3B). However, some correspondence between starvation resistance and life-span was seen (Fig. 3C). Increased SOD levels were seen in *chico*^{1} homozygotes but not in heterozygotes (*17*) (Fig. 4). Thus, modulation by IIS of longevity, and of SOD levels, has evidently been conserved between *C. elegans* and *Drosophila*. Furthermore, effects of this pathway on fertility are widespread (*1, 3, 18*). However, effects on stress resistance are not well conserved, nor do any of the above associated affects appear to be causal in extending life-span.

Our results raise the question of whether IIS regulates aging in mammals. Whereas both the *C. elegans* and *Drosophila* genomes contain a single insulin/IGF receptor, mammals possess distinct receptors for insulin and IGF-I, plus a third insulin receptor–like receptor of unknown function. Potentially, any or all of these receptors may play a role in regulating aging. Caloric restriction (CR), which increases life-span in rodents (*19*), and possibly primates (*20*), reduces circulating levels of both insulin and IGF-I (*21, 22*). In the case of IGF-I, there is further evidence for a role in the control of longevity (*22, 23*). Growth hormone (GH) acts via IGF-I to control mammalian body size, and circulating IGF-I levels correlate with body size in mice, dogs, and humans (*23, 24*). Furthermore, CR can reduce body size (*22*). In mice and dogs (and possibly humans), there is a marked negative correlation between body size and longevity (*24–26*). In addition, long-lived Ames hypopituitary mouse dwarves are deficient in GH and other pituitary hormones and have reduced circulating IGF-I (*27*). Mutation of the human equivalent of the Ames dwarf gene, *Prop-1*, also causes dwarfism and, possibly, delayed aging (*28*). The Laron dwarf mouse, which has no GH receptor and very low IGF-I levels, exhibits life-span increases of up to 55% (*29*).

Whereas the effects of *chico*^{1} on development that result in reduced body size are recessive, its effects on life-span are semidominant. This may reflect the noncatalytic and dosage-dependent nature of the function of CHICO as a docking protein. It has been proposed that reduced body size per se increases life-span in mammals (*23*). Alternatively, the same genes may independently regulate growth during the preadult period and regulate survival during the adult period. Our data support the latter interpretation because *chico*^{1} heterozygotes are long-lived, yet of normal size. Likewise, the effect of CR on aging may be observed in the absence of its effects on body size.

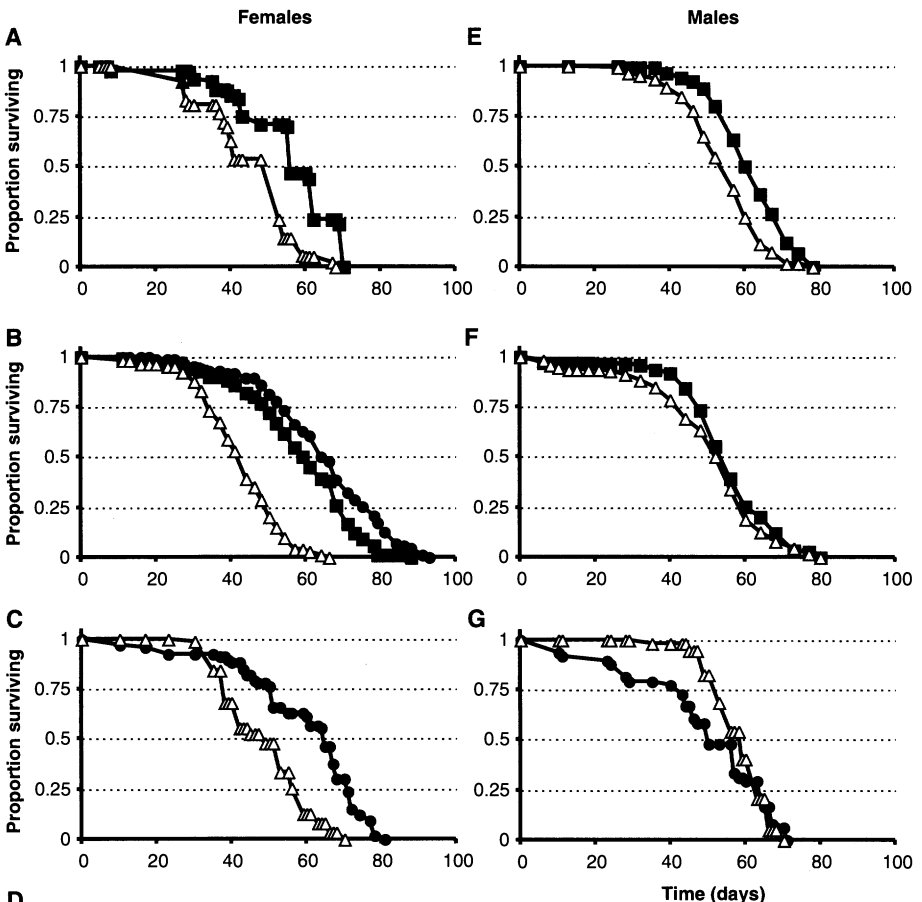

Fig. 1. Effect of *chico*^{1} on life-span. Median life-spans and sample sizes, respectively, for females are as follows. (**A**) +/+, 53 days and N = 119; +/*chico*^{1}, 56 days and N = 128. (**B**) +/+, 44 days and N = 105; +/*chico*^{1}, 60 days and N = 100; *chico*^{1}, 65 days and N = 86. (**C**) +/+, 49 days and N = 61; *chico*^{1}, 65 days and N = 67. (**D**) +/+, 46 days and N = 172; +/*chico*^{1}, 54 days and N = 171; *chico*^{1}, 58 days and N = 176. Data for males are as follows. (**E**) +/+, 54 days and N = 136; +/*chico*^{1}, 56 days and N =135. (**F**) +/+, 57 days and N = 104; +/*chico*^{1}, 62 days and N = 140. (**G**) +/+, 59 days and N = 54; *chico*^{1}, 50 days and N = 48. Statistical comparisons (log rank test) are as follows for females: +/*chico*^{1} versus +/+, $P < 0.0001$ (A, B, and D); *chico*^{1} versus +/*chico*^{1}, $P = 0.0008$ and <0.0001 (B and D, respectively); and *chico*^{1} versus +/+, $P < 0.0001$ (B, C, and D). Statistical comparisons (log rank test) are as follows for males: +/*chico*^{1} versus +/+, $P < 0.0001$ and < 0.0866 (not significant) (E and F, respectively). In all panels, circles represent *chico*^{1}/*chico*^{1}; squares represent +/*chico*^{1}, and triangles represent +/+.

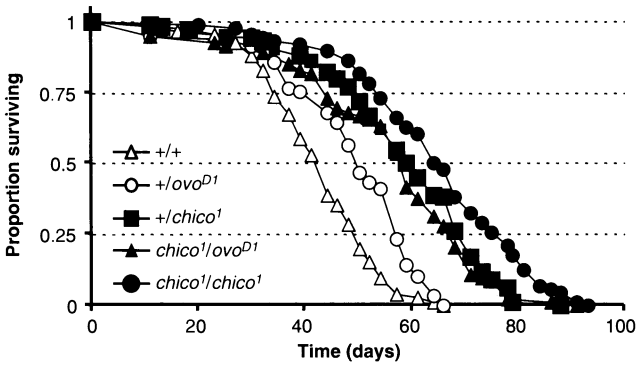

Fig. 2. Effects of *chico*^{1} and *ovo*^{D1} on female life-span. Median life-spans and sample sizes, respectively, are as follows: +/+, 44 days and N = 105; +/*ovo*^{D1}, 50 days and N = 90; +/*chico*^{1}, 60 days and N = 100; +/*chico*^{1} +/*ovo*^{D1}, 59 days and N = 93; and *chico*^{1}, 65 days and N = 86. Statistical comparisons (log rank test) are as follows: +/+ versus +/*ovo*^{D1}, $P < 0.0001$; +/*ovo*^{D1} versus +/*chico*^{1} +/*ovo*^{D1}, or +/*chico*^{1}, $P < 0.0001$; +/*chico*^{1} +/*ovo*^{D1} versus +/*chico*^{1}, no significant difference; and *chico*^{1} versus +/*chico*^{1} +/*ovo*^{D1} or +/*chico*^{1}, $P = 0.0008$ in each case.

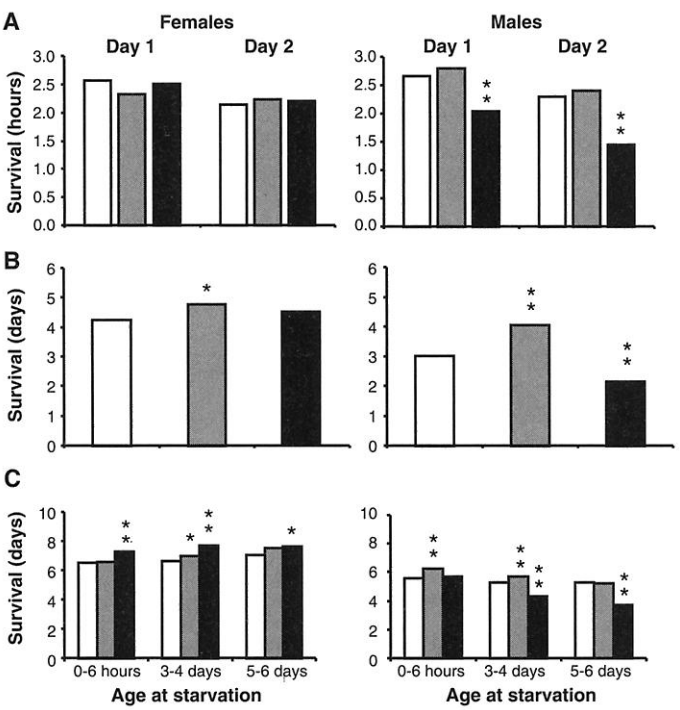

Fig. 3. Effect of *chico*^{1} on stress resistance. Histograms show mean survival time. Probability that values are identical to wild type is indicated as follows: *, $0.05 > P > 0.01$; **, $P < 0.01$ (log rank). (**A**) Heat stress resistance. Sample sizes for females were as follows: 49 (+/+), 50 (+/*chico*^{1}), and 51 (*chico*^{1}) on day 1 and 49 (+/+), 50 (+/*chico*^{1}), and 52 (*chico*^{1}) on day 2. Sample sizes for males were as follows: 50 (+/+), 50 (+/*chico*^{1}), and 50 (*chico*^{1}) on day 1 and 52 (+/+), 51 (+/*chico*^{1}), and 47 (*chico*^{1}) on day 2. (**B**) Oxidative stress (methyl viologen) resistance. Sample sizes for females were 100 (+/+), 100 (+/*chico*^{1}), and 99 (*chico*^{1}). Sample sizes for males were 99 (+/+), 100 (+/*chico*^{1}), and 69 (*chico*^{1}). (**C**) Starvation resistance. Sample sizes for females were as follows: from 0 to 6 hours, 150 (+/+), 150 (+/*chico*^{1}), and 150 (*chico*^{1}); from 3 to 4 days, 147 (+/+), 148 (+/*chico*^{1}), and 146 (*chico*^{1}); and from 5 to 6 days, 150 (+/+), 150 (+/*chico*^{1}), and 151 (*chico*^{1}). Sample sizes for males were as follows: from 0 to 6 hours, 150 (+/+), 150 (+/*chico*^{1}), and 101 (*chico*^{1}); from 3 to 4 days, 149 (+/+), 148 (+/*chico*^{1}), and 101 (*chico*^{1}); and from 5 to 6 days, 149 (+/+), 149 (+/*chico*^{1}), and 97 (*chico*^{1}). In all panels, white bars represent +/+, gray bars represent +/*chico*^{1}, and black bars represent *chico*^{1}/*chico*^{1}.

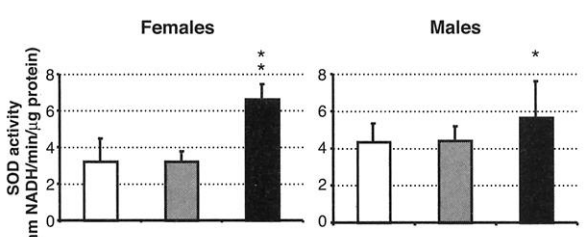

Fig. 4. Effect of *chico*^{1} on total superoxide dismutase (Cu/Zn and Mn SOD) activity. Histograms show mean values with 95% confidence intervals. Probability that the values are identical to that of the wild type is indicated as follows: *, $0.05 > P > 0.01$; **, $P < 0.01$ (paired t test). Replicate assays were conducted at several different times. Sample sizes for females were 6 (+/+), 6 (+/*chico*^{1}), and 4 (*chico*^{1}). Sample sizes for males were 5 (+/+), 6 (+/*chico*^{1}), and 4 (*chico*^{1}). White bars represent +/+, gray bars represent +/*chico*^{1}, and black bars represent *chico*^{1}/*chico*^{1}.

Together, our results with fruit flies and recent findings with nematodes and mice suggest that the role of IIS (perhaps IGF-I in mammals) in regulating longevity is evolutionarily conserved throughout the animal kingdom.

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10. D. J. Clancy *et al.*, data not shown.
11. *pCSR4-chico* 1.1 and 2.3 (insertions on the third chromosome) were generated in a base stock marked with *yellow*, *white* (*yw*). Females of the *chico*^{1} Dahomey stock were crossed either to males from each *pCSR4-chico* stock or to males from the *yw* base stock.
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