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

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Abstract: The Urosporia 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 influence the molecular mechanisms underlying aging and longevity. In Caenorhabditis elegans, mutation of the genesdaf-2 andage-1, which encode components of an insulin/IGF signaling pathway (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 Urosporia 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 DArk1) 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 chico1 (3) and PKB1 (9) homozygotes and InrE19/InrE19 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, PKB1 homozygotes and InrE19/InrE19 flies were short-lived. By contrast, chico1 extended life-span (Figure 1). Homozygous chico1 females exhibited an increase of median and maximum life-span of up to 48 and 41%, respectively. chico1 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 chico1 itself extended lifespan, we tested the effect on life-span of pCSR4-chico, a P element containing chico+. This construct fully rescues the dwarf phenotype of chico1 (3). chico1 was crossed to two stocks containing independent pCSR4-chico insertions (pCSR4-chico 1.1 and 2.3). As a control, chico1 was also crossed to the base stock in which the P element insertions were made. Pregeny with either two copies (chico1 heterozygotes with one chico transgene) or one copy (chico1 heterozygotes alone) of chico1 were compared (11). The rescue construct significantly reduced life-span relative to the +/chico1 control. The median female life-span of 54 days in +/chico1 was reduced to 46 days in +/chico1, +/pCSR4-chico 1.1 flies and 52 days in +/chico1, +/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 chico1 is a null allele, its effect on lifespan indicates that the wild-type chico gene acts to accelerate aging.

Of the mutations tested, only chico1 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, chico1 is not homozygous lethal, presumably because the INR receptor can signal to PI3K directly, as well as indirectly via CHICO (3). Thus, chico1 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 InrE19/InrE19 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 PKB1 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 chico1 was mediated by processes previously shown to affect aging. A reduction in fecundity extends life-span in Drosophila females (14, 15); chico1 heterozygous females have reduced fecundity, and the homozygotes are almost sterile (3, 12). To test whether the increased life-span of chico1 females was due to reduced fecundity, we examined the interactions between chico1 and the dominant, female-sterile mutant ovoD1. This mutation blocks oogenesis at stage 4, before vitellogenesis commences (16), and extends female life-span (15). If chico2 extends female life-span by the exact same mechanism as ovoD1, then the three sterile genotypes (chico1, +/ovoD1, and +/ovoD1 + chico1) should have similar life-spans and live longer than the subfertile chico1 heterozygotes. In fact,
the chico homozygotes lived significantly longer than all other genotypes (Figure 2). In addition, the partially fertile chico heterozygotes lived as long as the sterile flies that were heterozygous for both ovo and chico and lived significantly longer than the sterile ovo heterozygotes. The effect of chico on female life-span is therefore not a consequence of the same mechanism of reduced fecundity as is produced by ovo. If chico 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 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 flies to three stressors, but only one showed any correspondence with life-span (17) (Figure 3). No resistance to heat stress (37°C) was seen (Figure 3A). Slight resistance to oxidative stress (methyl viologen) was observed in chico heterozygotes but not in homozygotes (Figure 3B). However, some correspondence between starvation resistance and life-span was seen (Figure 3C). Increased SOD levels were seen in chico homozygotes but not in heterozygotes (17) (Figure 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 effects 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 hypophysectomy 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 dwarfish 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 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 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.

Figure 1. Effect of chico on life-span. Median life-spans and sample sizes, respectively, for females are as follows. (A) */+, 53 days and N = 119; */chico1, 56 days and N = 128. (B) */+, 44 days and N = 105; */chico1, 60 days and N = 100; chico0, 65 days and N = 86. (C) */+, 49 days and N = 61; chico0, 65 days and N = 67. (D) */+, 46 days and N = 172; */chico1, 54 days and N = 171; chico1, 58 days and N = 176. Data for males are as follows. (E) */+, 54 days and N = 136; */chico1, 56 days and N = 5135. (F) */+, 57 days and N = 104; */chico1, 62 days and N = 140. (G) */+, 59 days and N = 54; chico0, 50 days and N = 48. Statistical comparisons (log rank test) are as follows for females: */chico1 versus */+, P < 0.0001 (A, B, and D); chico0 versus */chico1, P = 0.0008 and 0.0001 (B and D, respectively); and chico0 versus */+, P < 0.0001 (B, C, and D). Statistical comparisons (log rank test) are as follows for males: */chico1 versus */+, P < 0.0001 and < 0.0866 (not significant) (E and F, respectively). In all panels, circles represent chico0; squares represent */chico1, and triangles represent */+.
Figure 2. Effects of chic01 and ovar01 on female lifespan. Median life-spans and sample sizes, respectively, are as follows: +/-, 44 days and N = 105; +/ovar01, 50 days and N = 90; +/- chic01, 60 days and N = 100; +/- chic01/+ ovar01, 59 days and N = 93; and chic01, 65 days and N = 86. Statistical comparisons (log rank test) are as follows: +/- versus +/ovar01, P < 0.0001; +/- ovar01 versus +/- chic01/+ ovar01, or +/- chic01, P < 0.0001; +/- ovar01 versus +/- chic01, no significant difference; and chic01 versus +/- chic01/+ ovar01 or +/- chic01, P = 0.0008 in each case.

Figure 3. Effect of chic01 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 (+/chic01), and 51 (chic01) on day 1 and 49 (+/+), 50 (+/chic01), and 52 (chic01) on day 2. Sample sizes for males were as follows: 50 (+/+), 50 (+/chic01), and 50 (chic01) on day 1 and 52 (+/+), 51 (+/chic01), and 47 (chic01) on day 2. (B) Oxidative stress (methyl viologen) resistance. Sample sizes for females were 100 (+/+), 100 (+/chic01), and 99 (chic01). Sample sizes for males were 100 (+/+), 100 (+/chic01), and 69 (chic01). (C) Starvation resistance. Sample sizes for females were as follows: from 0 to 6 hours, 150 (+/+), 150 (+/chic01), and 150 (chic01); from 3 to 4 days, 147 (+/+), 148 (+/chic01), and 146 (chic01); and from 5 to 6 days, 150 (+/+), 150 (+/chic01), and 151 (chic01). Sample sizes for males were as follows: from 0 to 6 hours, 150 (+/+), 150 (+/chic01), and 101 (chic01); from 3 to 4 days, 149 (+/+), 148 (+/chic01), and 101 (chic01); and from 5 to 6 days, 149 (+/+), 149 (+/chic01), and 97 (chic01). In all panels, white bars represent +/-, gray bars represent +/- chic01, and black bars represent chic01 chic01.

Figure 4. Effect of chic01 on total superoxide dismutase (Cu/Zn and Mn SOD) activity. Histograms show mean values with 95% confidence intervals. Probabilities 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 (+/chic01), and 4 (chic01). Sample sizes for males were 5 (+/+), 6 (+/chic01), and 4 (chic01). White bars represent +/-, gray bars represent +/- chic01, and black bars represent chic01 chic01.
Supplementary Material

Methods

Fly culture and handling
All flies were reared at standard low density, and virgins were collected and handled with CO₂ anesthesia. Adults of the same sex were kept at a density of 10 per vial. Flies were transferred to fresh medium, and deaths were scored three times per week. The survival of all genotypes was compared with the base stock by using log rank tests [R. G. Miller, Survival Analysis (Wiley, New York, 1981)].

The Dahomey stock used to outcross the mutations examined has been in laboratory culture for 30 years in population cages. Stocks cultured in these conditions retain adult survival rates comparable to those of fly stocks freshly collected from nature [C. Sgrò, L. Partridge, Am. Nat. 156, 341 (2000)]. Any positive effects of the mutations on adult survival could therefore not be attributed to rescue of lowered life-span caused by inbreeding or laboratory adaptation.

Biodemographic analysis
In order to investigate whether differences in life-span between IIS mutants resulted from increased initial (non-age-dependent) mortality or from changes in the rate of acceleration of mortality with increasing age, we analyzed patterns of age-specific mortality in these strains. The best fit to changes in age-specific mortality was with the Gompertz-Makeham model \( m(t) = A_0 e^{Gt} + M_0 \), which characterizes mortality profiles \( m(t) \) mortality rate at time \( t \) in terms of three variables: (i) an initial mortality rate (IMR, or \( A_0 \)), (ii) a mortality rate acceleration constant (the Gompertz coefficient, \( G \)), and (iii) the Makeham parameter \( (M_0) \), which is an age-independent mortality constant, distinct from \( A_0 \), which may reflect environmental effects. An increase in the IMR would indicate that the strain was merely sick, whereas an increase or decrease in the Gompertz coefficient is consistent with accelerated or retarded aging, respectively. Our most sensitive test, containing the most scoring intervals, is that shown in Fig. 1B of the report. Here, the maximum likelihood estimate of \( G \) was reduced from 0.2011 in controls (Dahomey) to 0.1455 in \( \text{chico}^1 \) homozygotes (\( P = 0.0493; \chi^2 \) value, derived from the difference between likelihood values of parameter-constrained and unconstrained models). Thus, \( \text{chico}^1 \) reduces the rate of aging. By contrast, the estimates of \( A_0 \) did not differ significantly between controls and \( \text{chico}^1 \) mutants, nor did either parameter differ from controls in \( \text{chico}^1 \) heterozygotes. In another trial, the parameter estimate in \( \text{Inr} \) transheterozygote females for \( G \) was reduced (\( P = 0.0091 \)), whereas that for \( A_0 \) was increased (\( P = 0.0080 \)) relative to controls. This suggests that these flies age more slowly but are also frailer than the wild type (see Web table 1).

Supplemental Table 1. Maximum likelihood estimate of Gompertz-Makeham parameters.

<table>
<thead>
<tr>
<th>Line</th>
<th>( A_0 )</th>
<th>( G )</th>
<th>( M_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.00115</td>
<td>0.18802</td>
<td>2.06e-9⁻⁻⁹</td>
</tr>
<tr>
<td>( \text{InrE}^{19\text{E}}/\text{InrGC}^{25\text{GC}} )</td>
<td>0.02158</td>
<td>0.08768</td>
<td>2.06e-9⁻⁻⁹</td>
</tr>
<tr>
<td>( \text{PKB}^3 )</td>
<td>0.00486</td>
<td>0.15060</td>
<td>2.06e-9⁻⁻⁹</td>
</tr>
</tbody>
</table>
This agrees with the results of Tatar et al. [see (13) in report]. In PKB\(^{1}\) homozygotes, parameter estimates did not differ from controls, although an increased \(A_0\) was borderline significant (\(P = 0.0664\)).

**Measurement of female fecundity**

Flies were reared at constant low density in bottles, collected as virgins, and placed individually in vials with food medium and live yeast paste. In one experiment, egg production between days 4 and 14 was monitored; in the second experiment, eggs between days 4 and 16 were counted. In both experiments, \(chico^1\) heterozygotes laid significantly fewer eggs than did the controls (first experiment mean = 231 versus mean = 305, \(P < 0.0001\), \(+/+\), \(N = 49\); \(+/chico^1\), \(N = 57\); second experiment mean = 469 versus mean = 566, \(P = 0.0152\), \(+/+\), \(N = 33\); \(+/chico^1\), \(N = 35\)).

**Measurement of resistance to heat stress, oxidative stress, and starvation**

For stress resistance experiments, mortality was monitored, mean survival times were calculated, and statistical analysis was performed by the nonparametric log rank test, unless otherwise stated. To measure resistance to heat stress, we placed 5- to 7-day-old virgins in a vial in single-sex groups (10 to a vial, with moistened filter paper and a cotton wool plug with an air-permeable plastic cap), then incubated them at 37°C in a water bath. Time to immobility was scored with independent groups of flies on two consecutive days. To measure resistance to oxidative stress, we put 3- to 4-day-old virgins in a vial (9 or 10 flies to a vial), with eight filter paper discs soaked with 500 μl of 5% sucrose and 20 mM methyl viologen (Paraquat). To measure starvation resistance, we placed 0-, 3-, and 5-day-old virgins in single-sex groups in vials with 1% agar and 0.3% methyl 4-hydroxybenzoate (Nipagin).

**Superoxide dismutase (SOD) assays**

To assay total SOD activity, we homogenized groups of 4-day-old virgins in 500 μl of 50 mM tris (pH 8.0) and 0.125 mM NaCl, then centrifuged them at 2000g for 5 min. The supernatant was centrifuged in a size cutoff microconcentrator (10,000 dalton, Centricon-10, Millipore Corporation) to remove peptides and small molecules with SOD-like activity. The protein retentate was diluted 30-fold in homogenizing buffer, then reconcentrated in the same manner. Soluble protein concentration was assayed with the bicinchoninic acid (BCA) protein assay (Pierce Company). Total SOD activity was measured by using the method of F. Paoletti and A. Mocali [Methods Enzymol. 186, 209 (1990)]. The oxidation of NADH depends on the availability of superoxide radicals in solution. NADH oxidation was monitored by decreasing \(A_{340nm}\). For a control, NADH oxidation was monitored for 20 min under superoxide-producing conditions. Statistical analysis was by paired \(t\) tests, pairing representing assays that were done on the same day.

If you have any questions, please contact David Gems (d.gems@galton.ucl.ac.uk) or Linda Partridge (l.partridge@ucl.ac.uk).