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A Mutant Drosophila Insulin Receptor Homolog That Extends Life-Span and Impairs Neuroendocrine Function

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The Drosophila melanogaster gene insulin-like receptor (InR) is homologous to mammalian insulin receptors as well as to Caenorhabditis elegans daf-2, a signal transducer regulating worm dauer formation and adult longevity. We describe a heteroallelic, hypomorphic genotype of mutant InR, which yields dwarf females with up to an 85% extension of adult longevity and dwarf males with reduced late age-specific mortality. Treatment of the long-lived InR dwarfs with a juvenile hormone analog restores life expectancy toward that of wild-type controls. We conclude that juvenile hormone deficiency, which results from InR signal pathway mutation, is sufficient to extend life-span, and that in flies, insulin-like ligands nonautonomously mediate aging through retardation of growth or activation of specific endocrine tissue.

Molecular similarity between fly InR and worm daf-2 suggests that mutants of InR in flies should affect adult life-span, as do mutants of daf-2 in worms. InR and daf-2 are members of the insulin receptor family with homology to mammalian insulin and insulin-like growth factor–1 (IGF-1) receptors (1, 2). Worms carrying temperature-sensitive mutations in daf-2 form dauers at high temperature, but at lower temperature develop directly into adults with extended longevity and resistance to exogenous stress (3, 4). Genotypes homozygous for mutant InR have been reported to be lethal (5, 6), but several heteroallelic combinations of InR alleles produce viable, dwarf adults that are slow to develop: InREC34/InRE19 and InRGC25/InRC25 [InRC25 reported in (5)], and InRE19/InR5545 (Table 1). In addition, InRE19/InRE19 was found to be viable and dwarf once crossed into a new isogenic background (7). Dwarf females eclosed with extremely immature ovaries, and the egg chambers of young adults remain previtellogenic (Fig. 1, A through C).

Measurement of INR kinase activity (8) indicated that the InR5545 and InRE19 alleles both confer loss of INR function (Fig. 2). Basal activity of heterozygotes +/InRE19 and +/InR5545 was 45% of that of the wild type. Insulin stimulation increased kinase activity of INR from +/+ and +/InRE19 flies by 60%, but only by 26% from +/InR5545 flies (P < 0.05, for insulin stimulation of +/InRE19 versus +/InR5545). Basal kinase activity of INR from InRE19/InRE19 and from InR5545/InR5545 flies was 14 and 11% of that of the wild type, respectively; neither were stimulated by insulin. InR5545 is a P-element insertion in exon-1 (5, 6); the molecular lesion of InRE19 has yet to be identified, but it does not appear to occur in the known coding region of the gene (9).

Life tables (10) of InR mutant adults were compared to concurrent cohorts of a wild-type isogenic strain (Fig. 3). Dwarfs of InREC34/InRE19 and InRGC25/InRE19 are short-lived. Dwarf InRE19/InRE19 and nondwarf +/InR5545 have moderately reduced survival; nondwarf +/InR5545 individuals are normal. In contrast, females of InR5545/InRE19 are 85% longer lived than wild-type controls and overall present reduced age-specific mortality. As described in the accompanying paper (11), the life-span of female D. melanogaster is also extended by mutation of the insulin receptor substrate homolog chico. Survivorship among male InR genotypes fol-

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Fig. 1. Ovaries and ovarioles of wild-type and InR dwarf flies, stained with rhodamine-phalloidin and oligreen. Bars in (A) through (D), 50 μm. (A) Wild-type ovariole prepared 4 hours after eclosion. Germarium and stage 1-3 egg chambers are present. (B) InR19/InR19 ovary 24 hours after eclosion. All ovarioles are immature. (C) InR19/InR19 ovariole 11 days after eclosion. The degree of egg chamber maturation resembles that of newly eclosed wild-type flies [compare to (A)]. (D) A representative ovariole of dwarf InRE19/InRE19 11 days after a single topical application of methoprene (1 μg) at 1 day after eclosion. The gradient of maturation is typical for normal, virgin females: a mature stage 14 egg is silhouetted on the right (arrow), while previtellogenic stage 1-6 egg chambers are distal [stages described in (35)]. (E) Effect of methoprene treatment on vitellogenesis in InR mutant females. Single applications of methoprene in 0.1 μl acetone solvent were made upon the abdomen of anesthetized flies at 1 day after eclosion (control, acetone only). Females were dissected at 11 days after eclosion (10 to 22 flies per group) and scored as vitellogenic if any ovariole was at or beyond stage 8. For continuous exposure, methoprene was volatilized in glass culture bottles for 10 days (four applications of 0.01 μg methoprene per bottle).
The fact that InR mutants are nonvitellogenic suggests a plausible mechanism for the extended longevity of InR<sup>5545</sup>/InR<sup>E19</sup> flies. Drosophila overwinter as adults in a reproductive diapause where egg development is arrested at previtellogenic stages (12, 13). In many insects, including Drosophila, reproductive diapause is proximally controlled through down-regulation of juvenile hormone (JH) synthesis by the corpora allata (CA) (14, 15). Ovaries of InR dwarf females morphologically resemble ovaries of diapause wild-type flies, and exogenous application of the JH analog methoprene to dwarf females initiated vitellogenesis (Fig. 1, C and D). Females of InR<sup>E19</sup>/InR<sup>E19</sup> responded to a single treatment of methoprene in a dose-dependent manner, but females of InR<sup>5545</sup>/InR<sup>E19</sup> required continuous exposure to hormone to induce any vitellogenesis (Fig. 1E). Direct assay of adult JH synthesis (16) verified that CA activity was reduced in InR dwarfs to about 23% of the wild-type level (Fig. 4). Because reduced JH synthesis is seen in

![Image](https://www.sciencemag.org/content/292/5526/1210/F1.large.jpg)

Fig. 2. Kinase activity of INR from membranes prepared from adult heads of defined genotypes. Membranes were solubilized and INR autophosphorylated in the absence or presence of insulin (1 μM), and immunoprecipitated with Ab dp1040. Each mean ± SEM represents data from three to six independent experiments for each genotype, each performed in triplicate.

Table 1. Phenotypes of InR dwarf and nondwarf genotypes relative to the coisogenic wild-type control (mean ± SEM).

<table>
<thead>
<tr>
<th>Genotype at InR</th>
<th>Development rate (days)*</th>
<th>Adult size (10&lt;sup&gt;−3&lt;/sup&gt; g)*</th>
<th>Fertility†</th>
<th>Life expectancy at eclosion [days (SEM)]</th>
<th>SOD activity V/v (10&lt;sup&gt;−2&lt;/sup&gt;)§</th>
<th>Triglycerides (μg/μg protein)¶</th>
<th>Volume O&lt;sub&gt;2&lt;/sub&gt; consumedµ</th>
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<tbody>
<tr>
<td>Dwarf</td>
<td></td>
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<tr>
<td>InR&lt;sup&gt;E19&lt;/sup&gt;/InR&lt;sup&gt;E19&lt;/sup&gt;</td>
<td>19.6 (0.38)</td>
<td>0.43</td>
<td>F: sterile</td>
<td>24.6 (0.98)</td>
<td>1.3</td>
<td>4.48</td>
<td>0.079</td>
</tr>
<tr>
<td>InR&lt;sup&gt;E19&lt;/sup&gt;/InR&lt;sup&gt;5545&lt;/sup&gt;</td>
<td>21.6 (0.53)</td>
<td>0.40</td>
<td>F: sterile</td>
<td>20.9 (0.79)</td>
<td>1.0</td>
<td>4.81</td>
<td>0.060</td>
</tr>
<tr>
<td>InR&lt;sup&gt;GC25&lt;/sup&gt;/InR&lt;sup&gt;E19&lt;/sup&gt;</td>
<td>21†</td>
<td>48%†</td>
<td>F: sterile</td>
<td>2.3 (0.53)</td>
<td>0.6</td>
<td>1.0</td>
<td>0.002</td>
</tr>
<tr>
<td>InR&lt;sup&gt;GC25&lt;/sup&gt;/InR&lt;sup&gt;79&lt;/sup&gt;</td>
<td>19†</td>
<td>56%†</td>
<td>F: sterile</td>
<td>1.7 (0.21)</td>
<td>0.6</td>
<td>1.0</td>
<td>0.002</td>
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<td>Nondwarf</td>
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<tr>
<td>InR&lt;sup&gt;5545&lt;/sup&gt;/InR&lt;sup&gt;+&lt;/sup&gt;</td>
<td>12.4 (0.18)</td>
<td>1.01</td>
<td>F: sterile</td>
<td>23.9 (1.1)</td>
<td>0.53</td>
<td>0.73</td>
<td>0.073</td>
</tr>
<tr>
<td>InR&lt;sup&gt;E19&lt;/sup&gt;/InR&lt;sup&gt;+&lt;/sup&gt;</td>
<td>11.8 (0.16)</td>
<td>1.02</td>
<td>M: 20%</td>
<td>30.5 (0.16)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.007</td>
</tr>
<tr>
<td>InR&lt;sup&gt;+&lt;/sup&gt;/InR&lt;sup&gt;+&lt;/sup&gt;</td>
<td>11.9 (0.13)</td>
<td>1.01</td>
<td>M: 35.9 (1.1)</td>
<td>0.01</td>
<td>0.18</td>
<td>0.005</td>
<td></td>
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</table>

* Development rate and adult body size were measured from the eclosion time and size of the first 10 adults of each sex; eggs were laid at constant density (50/vial). † Male fertility estimated as proportion of fertilized eggs produced by 20 males individually paired with wild-type females, and scaled to the fertility of wild-type males. § Enzyme activity measured by inhibition rate of substrate reduction (v) normalized by substrate reduction when Cu/Zn-superoxide dismutase (SOD) was inactivated (V); scaled by total protein. Estimates measured from five samples each of three females at 3 days old. ¶ Triglycerides per adult estimated from five samples each of three females at 3 days old. µ Volume of oxygen consumed (μL/mg/hour) measured from 100 flies (1:1 sex ratio) at 25°C over 4 hours in a closed 60-ml chamber. Values are the mean of four replicate measures per genotype made for cohorts between 2 to 8 days in age.
InR<sup>RE19</sup>/InR<sup>E19</sup> flies, which exhibit normal life-span, as well as in long-lived InR<sup>p545e</sup>/InR<sup>E19</sup> flies, the simple lack of JH may not be enough to extend longevity.

Loss of corpora allata JH accounts for dwarf infertility. Mutation of InR may increase longevity because infertility reduces allocation of metabolic resources to reproduction and frees resources for somatic maintenance (17) or because reduced JH in mutant flies induces specific physiological mechanisms of somatic persistence normally expressed during adult reproductive diapause. Adult D. melanogaster in reproductive diapause age at negligible rates and are stress resistant; these traits are reversed by treatment with methoprene (18). Extended survival is characteristic of adult reproductive diapause in acridid grasshoppers and in the monarch butterfly, and surgical ablation of the corpora allata to eliminate adult JH synthesis induces both diapause and increased longevity [reviewed in (19)]. Consistent with the notion that reduced JH synthesis can directly extend life-span, InR dwarf flies show somatic physiological changes (20) (Table 1): (i) triglycerides are elevated fourfold (F = 32.2, P < 0.001), as observed in diapause D. triauraria (21) and in dwarf D. melanogaster mutant for chico (22), and (ii) Cu/Zn-superoxide dismutase concentration is increased twofold (F = 9.42, P < 0.02), as is characteristic of long-lived mutants of Caenorhabditis elegans (23). Measured in young adults, no difference in mass-specific metabolic rate was detected (Table 1). We suggest that infertility need not be the direct cause of slowed aging in InR mutants; JH may simply control both fertility and life-span.

To test directly whether JH modulates survival in InR<sup>p545e</sup>/InR<sup>RE19</sup> female dwarfs, we investigated whether treatment with methoprene restores wild-type longevity to these mutants, even if it does not fully restore fertility (24). In concurrent trials of dwarf and wild-type flies, survival of methoprene-treated InR<sup>p545e</sup>/InR<sup>RE19</sup> females was reduced toward the level observed in isogenic controls (Fig. 5). This rescue is physiological rather than toxicological because, in wild-type controls, methoprene produced no significant change relative to ethanol-treated flies.

The InR pathway may alter endocrine function in two ways. Adult CA is derived from neurosecretory tissue of the larval brain gland. Adult dwarf CA may be immature upon metamorphosis as a result of cell autonomous effects of InR upon the development of neuroendocrine cells. A second way InR may alter endocrine function is that JH secretion by CA may be impaired by reduced neuropeptide transmission in the adult brain (25), due to a reduction of INR function in brain areas where it is normally expressed (26).

In C. elegans, the insulin/IGF-1 pathway influences dauer formation, fertility, and aging in part through nonautonomous, secondary signaling; sterility is not required for extended longevity in C. elegans because some long-liveddaf-2 are fully fertile (27). For Drosophila, we have shown that InR affects neurosecretory tissue specialized for secretion of juvenile hormone. Therefore, mutations in the insulin signaling pathway in flies autonomously affect cell proliferation, growth, and body size (5, 22), but nonautonomously affect diapause, reproduction, and life-span through effects upon specific neuroendocrine cells. Deficiency in a juvenoid-like hormone signal in worms and in flies may extend longevity because its absence leads to the inappropriate expression of parallel physiological programs normally reserved for dauer or diapause.

This invertebrate model may have parallels with mammalian aging. Ames and Snell mice are mutant for the genes Prop-l or Pit-1, respectively, and are defective for pituitary development [reviewed in (28)]. Consequently, they are deficient in growth hormone, prolactin, and thyroid-stimulating hormone, leading to hypothyroidism and presumably reduced synthesis of thyroxin, a retinoid hormone with potential functional similarity to JH (29). These mice are phenotypically dwarf, mildly obese, and long-lived (28). A remarkably similar phenotype is observed in mice lacking insulin receptor function in the central nervous system or those lacking the chico homolog, IRS-2, in all tissues: increased fat mass and infertility with accompanying neuroendocrine deficiency (30, 31). Although effects on life-span in these mice remain to be determined, the concordance of phenotypes suggests that insulin signaling may be central to a common mechanism that exists across taxa for the neuroendocrine regulation of metabolism and the reproductive state, and their associated consequences upon aging.

References and Notes
7. Balanced stocks of InR<sup>p545e</sup>, GCS2, EC43, InR<sup>E19</sup>, p5545 were provided by J. Jack (University of Connecticut Medical Center); a third-chromosomal isogenic strain marked with radiu incoplmens (r), red eye (y), and chico (c) was provided by K. Wharton (Brown University); and InR<sup>I</sup> in a wild-type background occurring in an isoeform line from Windsor, Canada, was provided by M. B. Sokolowski (University of Toronto).
8. Each InR mutant allele was backcrossed to the n irt strain for 10 generations by selection for e<sup>+,</sup> and
The assembly of higher order chromatin structures has been linked to the covalent modifications of histone tails. We provide in vivo evidence that lysine 9 of histone H3 (H3 Lys9) is preferentially methylated by the Clr4 protein at heterochromatin-associated regions in fission yeast. Both the conserved chromo- and SET domains of Clr4 are required for H3 Lys9 methylation in vivo. Localization of Swi6, a homolog of Drosophila HP1, to heterochromatic regions is dependent on H3 Lys9 methylation. Moreover, an H3-specific deacetylase Clr3 and a β-propeller domain protein Rik1 are required for H3 Lys9 methylation by Clr4 and Swi6 localization. These data define a conserved pathway wherein sequential histone modifications establish a “histone code” essential for the epigenetic inheritance of heterochromatin assembly.

The organization of the higher order chroma-
tin structure has been linked to the posttranslational modifications of histone tails, including acetylation, phosphorylation, and methy-
lацию (1). It has been suggested that distinct combinations of covalent histone modific-
auctions, also referred to as the “histone code,” provide a “mark” on the histone tails to recruit downstream chromatin-modifying pro-
teins (2, 3). This is best illustrated by recent studies indicating that the conserved bromo-
domain of several transcriptional coactivators bind specifically to acetylated lysine residues on histone tails (4, 5). The mechanisms re-
sponsible for the establishment and mainte-
nance of multiple covalent modifications within the same or different histone tail are not fully understood.

Modifications of histone tails have also been linked to heterochromatin assembly. Histones H3 and H4 are largely hypoacetylated in heterochromatin chromosomal regions in organisms as diverse as yeast, flies, and mammals (6–8). In fission yeast, hypoacety-
lation of histones is associated with the silent mating-type region and centromeres (9, 10), chromosomal domains that share many par-
allels with heterochromatic regions in higher eukaryotes (11). Centromeric regions com-
prising a central core of unique sequences surrounded by inner (imr) and outer (otr) repeats are assembled into silenced chromatin structures (12). Similarly, a large ∼15-kb chromosomal domain at the mating-type (mat2/3) region, including the mat2 and mat3 loci and an interval between them, known as the K-region, is maintained in a silent epige-
netic state (13, 14).

Recently, both Clr4 and its mammalian counter-
partner, SUV39H1, have been shown to have intrinsic histone H3-specific methyltrans-
ferase (HMTase) activity in vitro (19). How-
ever, it is not known whether histones are the physiologcal targets of these methy-
latransfers in vivo.

Consistent with previous findings, recombi-
nant Clr4 (rClr4) was found to contain HMTase activity exclusively for histone H3 (Fig. 1B). To identify the specific residue of H3 methylated by rClr4, we used synthetic peptides derived from the NH2-terminus of H3 as substrates in an in vitro HMTase assay (20, 21). Clr4 preferentially methylated the H3 1-20 unmodified peptide but failed to methylate the H3 19-35 unmodified peptide (Fig. 1C), indicating that the target residue of Clr4 HMTase resides in the first 20 amino acids of H3. To determine this target residue, we developed a synthetic H3 1-20 peptide set that contained covalent modifications on different amino acids. With these peptides as substrates, only acetyl or methyl modifications on Lys9 effectively blocked rClr4 HMTase activity, indicating that Clr4, like its mammalian homolog SUV39H1 (19), selectively methylates Lys9 of H3. Furthermore, similar to SUV39H1, rClr4 HMTase activity

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