

Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice

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ABSTRACT Recent evidence suggests that alterations in insulin/insulin-like growth factor 1 (IGF1) signaling (IIS) can increase mammalian life span. For example, in several mouse mutants, impairment of the growth hormone (GH)/IGF1 axis increases life span and also insulin sensitivity. However, the intracellular signaling route to altered mammalian aging remains unclear. We therefore measured the life span of mice lacking either insulin receptor substrate (IRS) 1 or 2, the major intracellular effectors of the IIS receptors. Our provisional results indicate that female *Irs1*^{-/-} mice are long-lived. Furthermore, they displayed resistance to a range of age-sensitive markers of aging including skin, bone, immune, and motor dysfunction. These improvements in health were seen despite mild, lifelong insulin resistance. Thus, enhanced insulin sensitivity is not a prerequisite for IIS mutant longevity. *Irs1*^{-/-} female mice also displayed normal anterior pituitary function, distinguishing them from long-lived somatotrophic axis mutants. In contrast, *Irs2*^{-/-} mice were short-lived, whereas *Irs1*^{+/-} and *Irs2*^{+/-} mice of both sexes showed normal life spans. Our results therefore suggest that IRS1 signaling is an evolutionarily conserved pathway regulating mammalian life span and may be a point of intervention for therapies with the potential to delay age-related processes.—Selman, C., Lingard, S., Choudhury, A. I., Batterham, A. L., Claret, M., Clements, M., Ramadani, F., Okkenhaug, K., Schuster, E., Blanc, E., Piper, M. D., Al-Qassab, H., Speakman, J. R., Carmignac, D., Robinson, I. C. A., Thornton, J. M., Gems, D., Partridge, L., Withers, D. J. Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. *FASEB J.* 22, 000–000 (2007)

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BIOLOGICAL AGING NOT ONLY LIMITS human life span but is also the major risk factor for a range of patho-

logical conditions with significant morbidity and mortality (1). Although aging appears to be stochastic in nature, involving accumulation of molecular damage caused by processes such as oxidation or glycation, the rate of aging is also influenced by genetic variation (2); there are striking differences in longevity between animal species, and mutations in single genes can extend life span in laboratory animals (2).

Although the mechanisms controlling the rate of aging in mammals remain poorly understood, there is growing evidence that the insulin/insulin-like growth factor (IGF) signaling (IIS) pathway is important (2, 3). This pathway has long been known to play pleiotropic roles in the development, growth, reproduction, stress resistance, and metabolism of multicellular animals (3). More recently it has been found to regulate adult life span in the nematode worm *Caenorhabditis elegans* and the fruit fly *Drosophila* (2, 4, 5). Evidence is mounting that it also does so in mice, in which heterozygote deletion of the Igf1 receptor (*Igf1r*) extends female life span by 25% (6), although others have criticized this study for the short life span in the control animals (7, 8). In addition, FIRKO mice, which lack the insulin receptor (*Insr*) in adipose tissue, display an 18% life span extension (9), although the mechanisms of life span extension in this model are uncertain. Modulation of IIS may also be the mechanism by which life span is extended in *Prop1*^{df}, *Pit1*^{dw}, and *Ghrhr*^{lit} mice and mice lacking the growth hormone (GH) receptor (10–13), all of which have reduced function of the somatotrophic axis and markedly decreased plasma IGF1 levels, but these models have additional endocrine abnormalities.

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In *C. elegans* and *Drosophila*, aging is controlled by activity of the insulin/IGF1 receptor, *via* the insulin receptor substrate (IRS)/phosphoinositide 3-hydroxy (PI3) kinase/forkhead transcription factor pathway (14, 15). However, in mammals, the situation is far more complex owing to greater signaling diversity with distinct insulin and Igf1 receptors and multiple IRS proteins, PI3 kinase signaling components, and forkhead proteins (16). Which of the postreceptor signaling molecules are the key regulators of life span remains unclear.

A further complication is that several mutations of IIS are clearly harmful. In *C. elegans* and *Drosophila*, certain mutations can result in developmental lethality, whereas others reduce adult life span, suggesting that moderate or specific alterations in pathway activity may be needed to extend life span (4). Similarly, severe global reduction of IGF1R and INSR function in humans or mice or deletion of *Insr* in selected tissues in mice can result in perinatal lethality, severe insulin resistance and type II diabetes, hyperlipidemia, liver dysfunction, and obesity (17–23). Thus, it seems likely that extension of the mammalian life span will only result from reductions of IIS of the right degree, involving the right intracellular signaling elements and in the right tissues (24). Furthermore, in view of the adverse effects of some IIS mutations, it is important to determine whether extension of life span is accompanied by an improvement in health during aging, an issue not addressed in the two mouse studies of IIS and life span thus far reported (6, 9).

Previously we have shown that deletion of *chico*, the single *Drosophila* IRS protein, extends life span by up to ~50% (4). To address the role of post-IIS receptor signaling in the regulation of mammal life span and age-related pathology, we therefore examined longevity in mice mutant for either *Irs1* or *Irs2*, the two major mammalian IRS proteins, and subsequently assessed metabolism and measures of age-sensitive markers of aging.

MATERIALS AND METHODS

Reagents

All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Mouse models and husbandry

The generation and genotyping of *Irs1* and *Irs2* mutant mice have been described previously (25, 26). *Irs1*^{-/-}, *Irs2*^{-/-}, *Irs1*^{+/-}, *Irs2*^{+/-}, and wild-type (WT) control littermate mice for each line were generated from heterozygote breeding pairs for either *Irs1* or *Irs2* strains (25, 26). All mouse strains were maintained on a C57BL/6 background after 10 backcrosses.

Longevity study

Mice were maintained at ~22°C under a 12-h light/dark cycle (lights on from 7:00 AM–7:00 PM). Mice were housed in

groups of three to eight same-sex littermates under specific pathogen-free conditions within individually ventilated cages (Techniplast UK Ltd, Kettering, Northamptonshire, UK). Mice were maintained in the same room for the duration of the study, and no additional animals were brought into this room; therefore, the colony was effectively kept under closed barrier conditions. Sentinel animals were screened every 3 months for pathogenic bacteria, viruses, and parasites, with all test results being negative throughout this study. Mice had *ad libitum* access to normal chow [2018 Teklad Global (5% fat, 18% protein, 57% carbohydrate, and 20% other components) Rodent Diet; Harlan Teklad, Bicester, Oxfordshire, UK] and water. Mice were monitored daily and weighed monthly, but otherwise were left undisturbed until they died naturally. If death appeared imminent, mice were weighed, examined for macroscopic pathological changes, and phenotypically assessed according to UK Home Office License Guidelines for animal welfare, and, if deemed necessary, individual mice were euthanized and this was considered the date of death. Of all the mice that died, 11% were euthanized, and the remainder died spontaneously. There were no significant differences in the proportions of each mode of death across the genotypes. When a sole surviving female mouse from each strain remained within a cage, this mouse was placed in an adjacent cage of female mice of the same strain. Sole surviving male mice within a cage were provided with a female companion not associated with this study. Kaplan-Meier survival curves were constructed using the known birth and death dates of each individual mouse, and the log-rank test was used to evaluate statistical differences between groups. Maximum life span was calculated as the mean age of the oldest 20% of mice from each genotype. Survival was assessed from a total of 81 female (46 *Irs1*^{+/-}, 14 *Irs1*^{-/-}, and 21 *Irs1*^{+/+}) and 124 male (79 *Irs1*^{+/-}, 10 *Irs1*^{-/-}, and 35 *Irs1*^{+/+}) *Irs1* mice, with 8 *Irs1* male mice (3 *Irs1*^{+/-}, 3 *Irs1*^{-/-}, and 2 *Irs1*^{+/+}) remaining alive at the time of this report and used in the analysis as censored observations. A total of 104 female (60 *Irs2*^{+/-}, 14 *Irs2*^{-/-}, and 30 *Irs2*^{+/+}) and 62 male (27 *Irs2*^{+/-}, 14 *Irs2*^{-/-}, and 21 *Irs2*^{+/+}) *Irs2* mice were used to generate the *Irs2* survival analysis, with 1 female (*Irs2*^{+/+}) and 2 male (*Irs2*^{+/-}) mice remaining alive and censored. All procedures were performed in accordance with the 1986 UK Home Office Animal Procedures Act and University College London, UK, Animal Ethical Committee guidelines.

Metabolic studies and analysis of food intake

For phenotyping studies, two separate cohorts of female *Irs1*^{-/-} mice and control littermates were generated from the same breeding pairs used in the longevity studies but not included in the longevity analysis. These mice were studied at 450 and 700 days of age. Blood samples were collected from mice *via* tail vein bleeds or from cardiac puncture on terminally anesthetized mice. Blood glucose was measured using a Glucometer Elite (Bayer Corporation, Tarrytown, NY, USA). Glucose, insulin, and IGF1 tolerance tests were performed on mice as described previously (25, 26). Fasting plasma insulin and leptin levels were determined with ELISAs (Linco Research, St. Charles, MO, USA) after an overnight fast. For food intake studies, mice were housed singly and allowed to acclimatize for 1 week before food intake was measured. Food intake and body mass were determined over 14 consecutive days. Resting metabolic rate (RMR) and core body temperature, measured using a rectal probe (Digitron Instrumentation Ltd., Torquay, Devon, UK), were determined as described previously (27). Individual mice from these additional cohorts were killed at either 450 or 700 days of age for further hormonal, anatomical, and gene expression analyses.

Pituitary hormone and plasma IGF1 assays

Pituitary glands were dissected and homogenized in 1 ml of phosphate-buffered saline. Aliquots were taken for assay of GH, prolactin, luteinizing hormone (LH), and thyroid-stimulating hormone (TSH) using specific reagents kindly provided by Dr. A. L. Parlow and by the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA (28). For IGF1 assays, plasma samples were extracted with acid/alcohol for 30 min at room temperature and then centrifuged. IGF1 was measured by radioimmunoassay using rat IGF1 (Diagnostic Service Laboratories Inc., Webster, TX, USA) as standard and for Iodogen labeling and goat anti-rat IGF1 antibody (Diagnostic Service Laboratories Inc.).

Pancreatic immunocytochemistry and measurement of β -cell mass

Pancreases were removed, cleared of fat and lymph nodes, fixed in Bouin's solution, embedded in paraffin, and cut into 5- μ m sections. Insulin staining and morphometric analysis were performed as described previously (27).

Real-time polymerase chain reaction (PCR)

Real-time PCR was performed as described previously using FAM/TAMRA primers from Applied Biosystems (Foster City, CA, USA) (27, 29). Analysis was performed using the standard curve method with results expressed relative to *Gapdh*. We used a general linear model (GLM) to examine the effects of genotype, age, and presence/absence of a genotype \times age interaction for real time-PCR data. Primer sets used were *Cat* Mm00437992_m1, *Cdkn2a/p16INK4a* Mm00494449_m1, *Cyp2d22* Mm00530542_m1, *Ercc8* Mm00522563_m1, *Fmo1* Mm00515795_m1, *Foxo1* Mm00518465_m1, *Gadd45b* Mm00435123_m1, *Gapdh* Mm99999915_g1, *Gnmt* Mm00494689_m1, *Gsta4* Mm00494803_m1, *Hspb1* Mm00834384_g1, *Igf1* Mm00833447_m1, *Mat1a* Mm00522563_m1, *Irs2* (MIRS 396412), *Mfn1* Mm00612599_m1, *Nox4* Mm00479246_m1, *Nrf1* Mm00447996_m1, *Pbeif1/Visfatin* Mm00451938_m1, *Pgc1a* Mm00731216_s1, *Ppara* Mm00440939_m1, *Sirt1* Mm01168521_m1, *Sod2* Mm00449726_m1, and *Tfam* Mm00447485_m1.

Generation, normalization, and analysis of microarray data

For the liver mRNA extraction, samples from female *Irs1*^{-/-} mice and control littermates were homogenized on ice using a Positron homogenizer in TRIzol reagent (Invitrogen Ltd, Paisley, UK), followed by purification using RNeasy columns (<http://www1.qiagen.com>; Qiagen Ltd, Crawley, UK). RNA quality and concentration were determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Changes in transcript abundance were measured using mouse whole genome oligonucleotide microarrays (Mouse Genome 430 2.0 Array; Affymetrix UK Ltd., High Wycombe, UK), with all protocols undertaken at the Institute of Child Health (ICH) Gene Microarray Centre, University College London (London, UK). A total of four biological replicates were used. The cRNA probe generation, washing, labeling, and scanning followed Affymetrix and ICH Gene Microarray Centre standard protocols. Raw image files were converted to probe-level data files (.cel files). Analysis of microarray data was limited to probesets with present transcripts (30), and the data were normalized with GCRMA at the probe level (30) and loess at the probeset level using functions available in BioConductor in R ([\[r-project.org\]\(http://www.r-project.org\)\) \(31\). Cyber-T \(32\) was used to generate *P* values for identification of significantly differentially expressed probesets. All probesets were aligned to Ensembl genes \(33\), and only unique probeset-to-gene alignments were used for further analysis. Functional annotation was taken from Ensembl \(33\).](http://www.</p></div><div data-bbox=)

Identification of regulated pathways from microarray data

To identify Gene Ontology (GO) (34) terms and functional annotation based on Interpro protein domains (35), we used Catmap (36) on gene lists based on ranks of Cyber-T statistics. The method uses a Wilcoxon rank-sum test to identify regulated categories.

Measurement of T cell surface marker expression

Venous blood from mice of the indicated ages and genotypes was analyzed for the naive/memory CD4 and CD8 T cell subsets by flow cytometry using the following antibodies: CD4 (GK1.5; eBiosciences, London, UK) CD8 α (53-6.6; BD PharMingen, Oxford, UK), CD44 (IM7; eBiosciences), and CD45RB (C363.16A; eBiosciences). Cells were acquired with a BD Biosciences LSR II instrument and analyzed using the FlowJo (Tree Star, Ashland, OR, USA) software package.

Computed tomography (CT) scanning

Formalin-fixed femurs were scanned using a Skyscan 1072 X-ray Microtomograph (SkyScan, Kontich, Belgium). Images were obtained with a rotation step of 0.67° between each image and were then reconstructed to give a stack of two-dimensional images, using NRecon (version 1.4.4; SkyScan). Three-dimensional modeling and analysis were then performed using CTAn software (version 1.5.0.2; SkyScan). The following bone morphometric parameters were obtained: percent cancellous bone volume, trabecular number, and trabecular separation.

Rotarod assay

Mice were placed on the Rotarod (RotaRod type 7650; Ugo Basile, Comerio, Italy) as it was rotating at 20 rpm. After 100 s the rate of revolution was increased and reached a maximum of 36 rpm within 90 s. The length of time that each animal spent on the rod was measured, with a cutoff time of 5 min. The test was performed three times for each animal with at least 15 min between each test.

Statistical methods

All statistical analyses were performed with Minitab (version 13; Minitab Ltd., Coventry, UK) and GraphPad Prism (version 4; GraphPad, San Diego, CA, USA) software, using unpaired *t* test, one-way analysis of variance, or a GLM. Results are reported as means \pm SE except where indicated. *P* < 0.05 was regarded as statistically significant.

RESULTS

Deletion of *Irs1* but not *Irs2* extends life span in mice

Log-rank testing was used to evaluate differences in life span in WT, heterozygote (+/-), and null (-/-) *Irs1* and *Irs2* mice. By using the data for both sexes combined, median life span in *Irs1*^{-/-} mice was signifi-

cantly increased by 140 days (from 760 to 900 days) or 18% relative to that of WT mice (log-rank test, $\chi^2=14.5$, $P<0.0001$) (Fig. S1; Tables S1 and S2). Analysis of each sex separately showed that the median life span in female *Irs1*^{-/-} mice was significantly increased, by 233 days (from 738 to 971 days) or 32% relative to that of WT mice (log-rank test, $\chi^2=12.5$, $P<0.001$) and was also greater than in female *Irs1*^{+/-} mice ($\chi^2=5.4$, $P<0.05$) (Fig. 1A; Tables S1 and S2). However, deletion of *Irs1* in male mice had no significant effect on life span compared with that for WT or *Irs1*^{+/-} mice ($P>0.05$, Fig. 1B; Tables S1 and S2), possibly explained by the lower numbers of male *Irs1*^{-/-} mice used in this study. In both female and male *Irs2*^{-/-} mice, life span was significantly reduced compared with that for WT controls ($\chi^2=14.1$, $P<0.001$ and $\chi^2=43.1$, $P<0.0001$ for

females and males, respectively) (Fig. 1C, D; Tables S1 and S2). No change in life span was seen in either female or male mice heterozygous for either *Irs1* or *Irs2* compared with WT controls (Tables S1 and S2). In summary, deletion of *Irs1* increased longevity in females but not in males, whereas homozygous deletion of *Irs2* significantly shortened life span in females and particularly in males, consistent with the progressive and sexually dimorphic diabetic phenotype seen in this strain.

Long-lived female *Irs1*^{-/-} mice show delayed age-sensitive markers of aging

Female *Irs1*^{-/-} mice appeared healthier than WT animals at older ages and had younger looking fur and

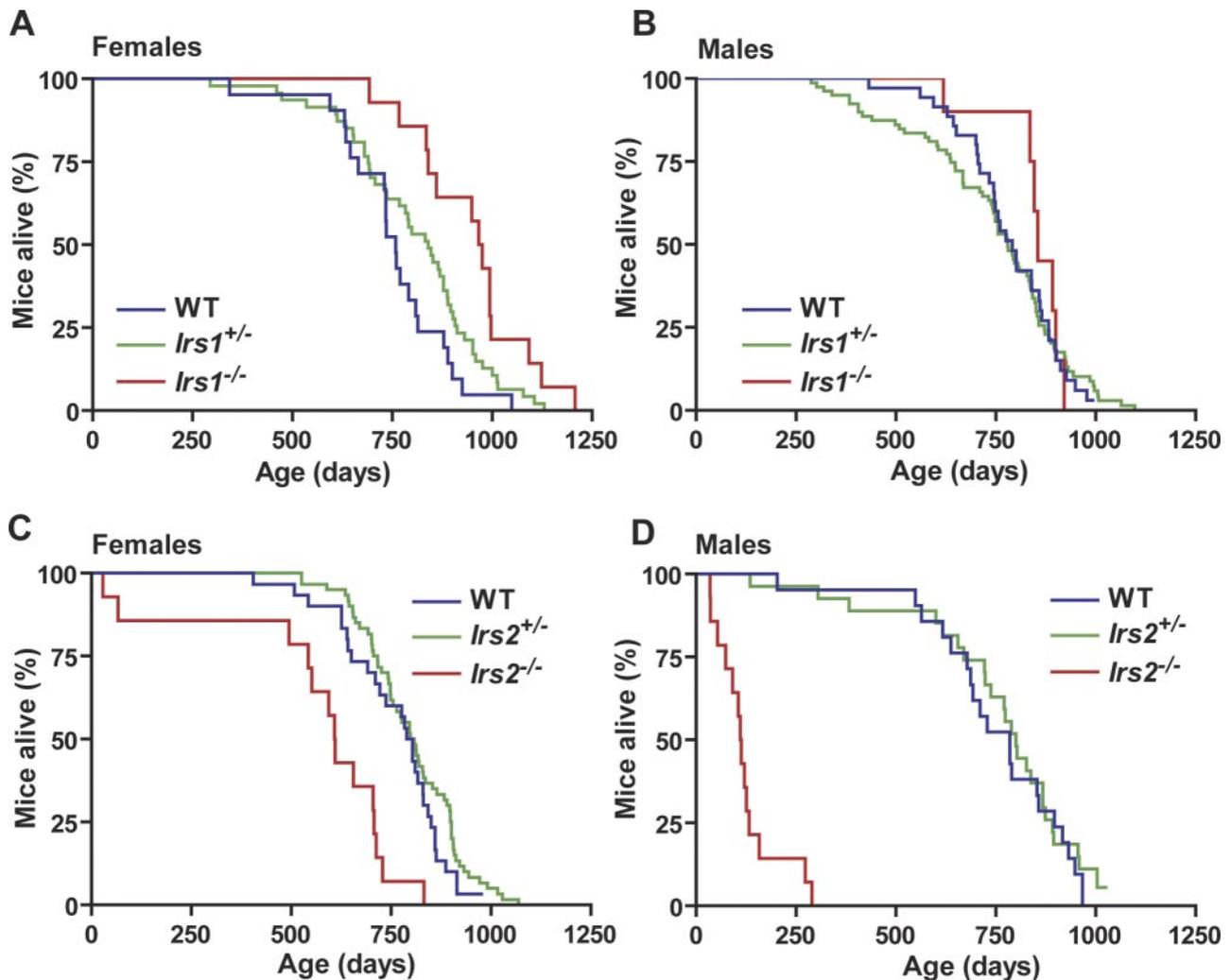


Figure 1. Female *Irs1*^{-/-} mice have increased life span. Kaplan-Meier survival curves are shown for WT, heterozygote (+/-), and null (-/-) mice for the female *Irs1* strain (A), the male *Irs1* strain (B), the female *Irs2* strain (C), and the male *Irs2* strain (D), with *P* values calculated using the log-rank test. A significant extension in median life span was observed in *Irs1*^{-/-} female mice compared with WT and +/- mice ($\chi^2=10.86$, $P<0.01$), but differences between genotypes in male *Irs1* mice did not reach significance ($\chi^2=1.07$, $P>0.05$). In *Irs2* mice, a significant difference in median life span was observed between WT, +/-, and -/- mice in both female ($\chi^2=29.35$, $P<0.0001$) and male mice ($\chi^2=78.23$, $P<0.0001$). Survival was assessed from 81 female (46 *Irs1*^{+/-}, 14 *Irs1*^{-/-}, and 21 *Irs1*^{+/+}) and 124 male (79 *Irs1*^{+/-}, 10 *Irs1*^{-/-}, and 35 *Irs1*^{+/+}) *Irs1* mice. Eight *Irs1* male mice (3 *Irs1*^{+/-}, 3 *Irs1*^{-/-}, and 2 *Irs1*^{+/+}) were alive at the time of this report and used in the analysis as censored observations; 104 female (60 *Irs2*^{+/-}, 14 *Irs2*^{-/-}, and 30 *Irs2*^{+/+}) and 62 male (27 *Irs2*^{+/-}, 14 *Irs2*^{-/-}, and 21 *Irs2*^{+/+}) *Irs2* mice were used, with 1 female (1 *Irs2*^{+/+}) and 2 male (2 *Irs2*^{+/-}) alive and censored. Red indicates null mice; green indicates heterozygote mice, and blue indicates WT mice in all cases.

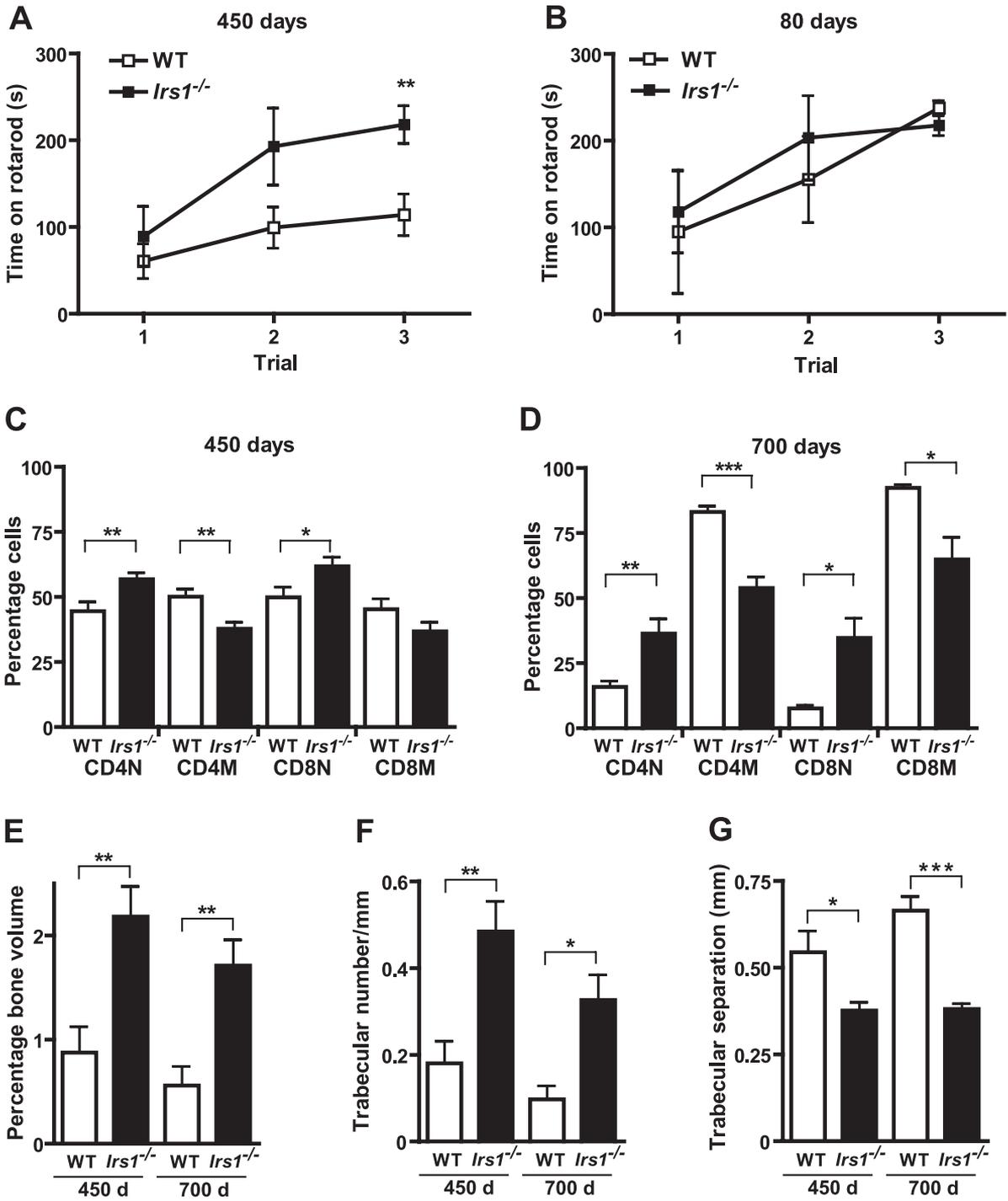


Figure 2. Delayed biomarkers of aging in female *Irs1*^{-/-} mice. *A, B*) Rotarod assays were performed with 450- and 80-day-old female WT and *Irs1*^{-/-} mice. Results presented are the mean length of time spent on the Rotarod (three trials per animal; *n*=5 per genotype). *C, D*) T cell subset analysis for naive (CD4N and CD8N) and memory (CD4M and CD8M) subsets in 450-day-old (*C*) and 700-day-old (*D*) female WT and *Irs1*^{-/-} mice (*n*=6 per genotype). *E, F, G*) Micro-CT analysis of femurs from 450- and 700-day-old female WT and *Irs1*^{-/-} mice. Percent cancellous bone volume (*E*), trabecular number (*F*), and trabecular separation (*G*) were determined (*n*=4 per genotype). Results are means ± SE. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

skin. Dermatitis, often ulcerative, occurs in old C57BL/6 mice, and its incidence is significantly reduced by caloric restriction (CR) (37). Our female *Irs1*^{-/-} mice were fully resistant to this pathological change, whereas WT mice showed rates similar to those seen previously (37) [incidence of dermatitis: 7 of 21

(33%) female WT and 0 of 14 (0%) female *Irs1*^{-/-} mice]. Using Rotarod assays to examine effects on age-related changes in motor coordination, we observed that at 450 days of age, older female *Irs1*^{-/-} mice performed better than WT littermates (**Fig. 2A**). This difference was not apparent at younger ages,

suggesting that improved performance was due to a delayed age-related decline in motor/neurological function and not the dwarf phenotype (Fig. 2B). We also examined age-related changes in immune function, which include an increase in memory T cells and a reduction in naive T cells (38). Such alterations have been shown to predict longevity phenotypes in mice (39). At both 450 and 700 days of age, female *Irs1*^{-/-} mice had significantly fewer memory and more naive T cells, and these differences were more pronounced in the older cohort (Fig. 2C, D). In C57BL/6 mice, cancellous bone volume decreases markedly between 6 wk and 2 yr of age (40). Micro-CT scanning of femurs from 450- and 700-day-old animals demonstrated increased cancellous bone volume with increased trabecular numbers and reduced trabecular separation in female *Irs1*^{-/-} mice compared with WT mice (Fig. 2E–G) despite the observation that young *Irs1*^{-/-} mice have osteopenia (41). Therefore, *Irs1*^{-/-} female mice showed improved health and delayed aging biomarkers compared with control animals at later ages across a range of indices.

We did not detect dermatitis in male *Irs1*^{-/-} mice [incidence of dermatitis: 7 of 35 (18%) in male WT and 0 of 10 (0%) in male *Irs1*^{-/-} mice]. Examination of T cell profiles in 450-day-old male *Irs1*^{-/-} mice revealed a reduced proportion of memory cells and increased numbers of naive cells compared with aged control animals (Fig. S1). Therefore, although we did not demonstrate life span extension in male *Irs1*^{-/-} mice most likely due to the small numbers of mice studied, these animals also show delayed aging biomarkers.

Female *Irs1*^{-/-} mice have persistent insulin resistance with preserved β -cell mass

Lowered fasting insulin and glucose levels have been reported during CR and in *Prop1*^{dk}, *Pit1*^{dw}, *Ghrhr*^{lit}, and GH receptor null mice (7, 8, 42). FIRKO mice also display both reduced insulin levels and resistance to age-related loss of glucose homeostasis (9). These findings suggest that improved insulin sensitivity might extend life span in these animals. Young (80-day-old) female C57BL/6 *Irs1*^{-/-} mice displayed normal fasting glucose levels, mildly impaired glucose tolerance, and mild hyperinsulinemia (data not shown), consistent with our previous studies on a mixed 129Sv/C57BL/6 background (25, 26). Between 450 and 700 days, there was deterioration in glucose homeostasis in WT but not in *Irs1*^{-/-} mice (Fig. 3A–C). *Irs1*^{-/-} mice at 450 days of age were hyperinsulinemic and were insulin resistant, as shown by insulin tolerance testing (Fig. 3D, E). In 700-day-old *Irs1*^{-/-} mice, insulin levels remained mildly elevated and increased β -cell mass was maintained compared with that in control mice, consistent with lifelong insulin resistance (Fig. 3D, F). These data demonstrate that improved insulin sensitivity is not a requirement for increased life span in IIS mutants.

Female *Irs1*^{-/-} mice do not have a pituitary endocrine phenotype

Irs1^{-/-} mice, like long-lived pituitary dwarf mice, show reduced body mass (Fig. 4A). However, levels of pituitary GH, TSH, LH, and prolactin in female *Irs1*^{-/-} mice did not differ from those of controls at any age (Fig. 4B; Fig. S2; data not shown); thus, unlike pituitary dwarf mice, these mice show no pituitary endocrine defects (7). In the pituitary dwarf mice and GH receptor null mice, reduced GH levels or action results in IGF1 deficiency (7). In contrast, and consistent with the preservation of GH function, circulating total IGF1 levels in female *Irs1*^{-/-} mice were the same as those in WT mice at all ages (Fig. 4C). However, glucose clearance in response to IGF1 in 450-day-old female *Irs1*^{-/-} mice was impaired, indicating IGF1 resistance (Fig. 4D)

Female *Irs1*^{-/-} mice are not calorically restricted but have reduced fat mass

CR extends life span in a range of organisms (42). Daily food intake was not reduced in female *Irs1*^{-/-} mice and, in fact, when adjusted for body weight, exceeded that of controls by 30% (Fig. 4E, F). However, by using a GLM with food intake as the dependent variable, genotype as a fixed factor, and body mass as a covariate no significant effect of genotype on food intake was observed (GLM, genotype effect: $F = 1.555$, $P = 0.226$; body mass effect: $F = 0.567$, $P = 0.459$). Although CR in mammals reduces core body temperature, rectal core body temperature was slightly elevated in female *Irs1*^{-/-} mice and significantly elevated at 450 days of age (Fig. 4G). However, indirect calorimetry revealed that at 450 and 700 days of age, mass-adjusted RMR was the same as that in female *Irs1*^{-/-} mice and WT animals (Fig. S1). Together, these findings imply that altered pituitary function, IGF1 levels, food intake, reduced core body temperature (43), and energy expenditure do not underlie the longevity phenotype in female *Irs1*^{-/-} mice. However, like mice under CR, *Irs1*^{-/-} mice displayed reduced fat mass throughout life (Fig. 4H). Relative to body weight, this reduction in fat mass was nonsignificant in 450-day-old animals but became significant at 700 days (Fig. 4I). This lower adiposity was reflected in reduced plasma leptin levels at both ages (Fig. S2).

Differential gene expression in the livers of female *Irs1*^{-/-} and WT mice

To further explore possible mechanisms underlying the increased life span in *Irs1*^{-/-} female mice, we undertook candidate mRNA expression analysis using liver tissue to permit comparison with similar data generated from other long-lived mutant mice and from mice under CR. In female *Irs1*^{-/-} and WT mice at 80, 450, and 700 days of age, there was evidence of alterations in expression of genes involved in oxidative stress, detoxification, DNA repair, and the response to

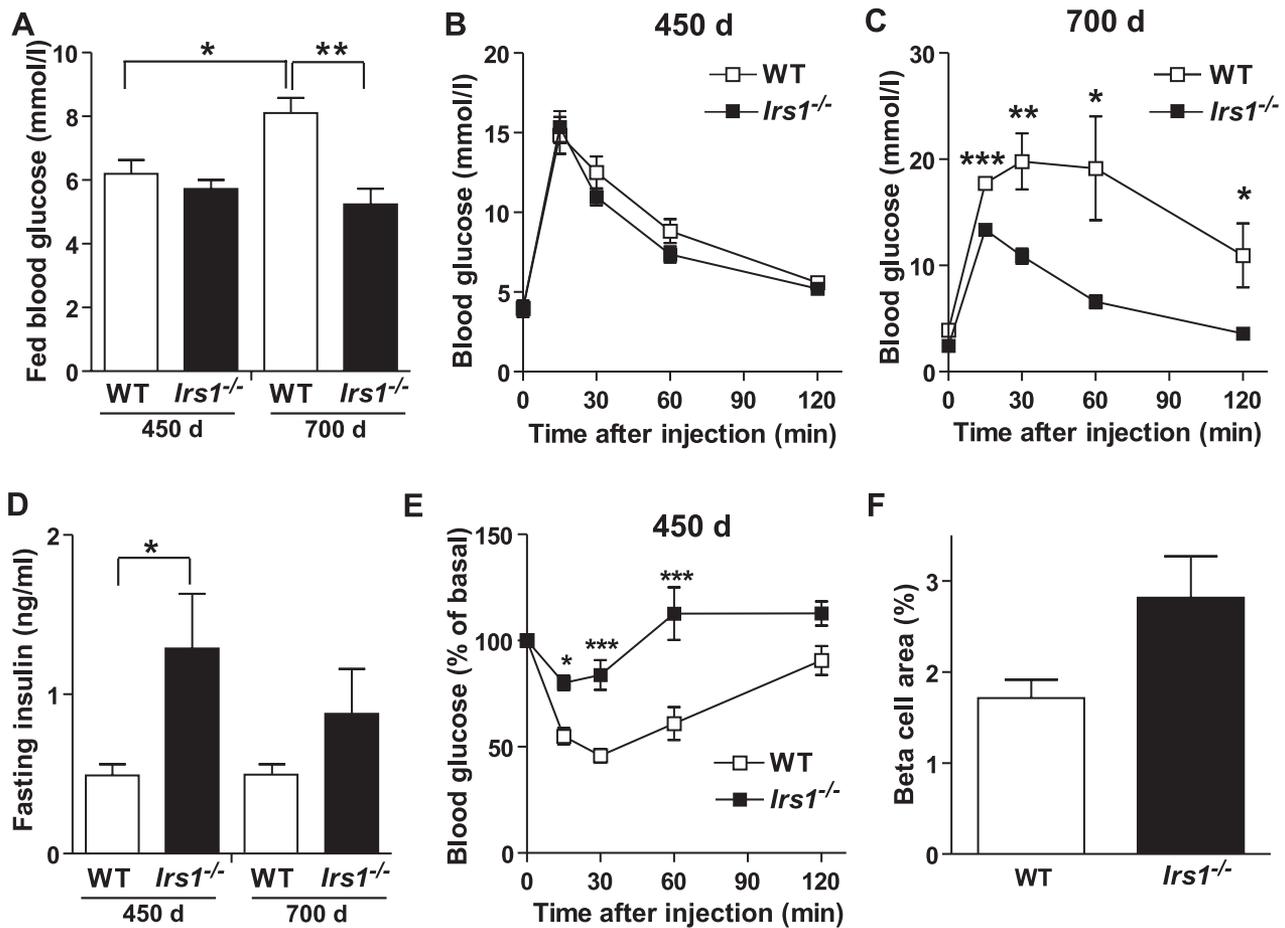


Figure 3. Female *Irs1*^{-/-} mice display persistent insulin resistance but preserved β -cell mass and have altered expression of candidate longevity assurance genes. **A)** Fed blood glucose levels in 450- and 700-day-old female WT and *Irs1*^{-/-} mice ($n=6-8$ per genotype). **B, C)** Glucose tolerance tests in 450-day-old (**B**) and 700-day-old (**C**) female WT and *Irs1*^{-/-} mice ($n=6-8$ per genotype). **D)** Fasting insulin levels in 450- and 700-day-old female WT and *Irs1*^{-/-} mice ($n=6$ per genotype). **E)** Insulin tolerance tests in 450-day-old female WT and *Irs1*^{-/-} mice (results are expressed as % glucose level at the beginning of the test; $n=6$ per genotype). **F)** β -cell area in 700-day-old female WT and *Irs1*^{-/-} mice (results are expressed as a percentage of pancreatic area; $n=4$ per genotype). Results are means \pm SE. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

genotoxic stress. An age-dependent reduction in catalase (*Cat*) expression was seen in WT mice by 700 days of age, whereas expression did not decline in female *Irs1*^{-/-} mice (Fig. S3). Expression of glutathione *S*-transferase a4 (*Gsta4*) was elevated in young *Irs1*^{-/-} mice and declined with age in animals of both genotypes (Fig. S3). Expression of excision repair cross-complementing rodent repair deficiency complementation group 8 (*Ercc8*), the Cockayne syndrome type A gene product, was elevated at all ages in *Irs1*^{-/-} mice (Fig. S3). A similar trend in expression values was seen with growth arrest and DNA damage inducible, β (*Gadd45b*) with the *Irs1*^{-/-} mice showing increased expression (Fig. S3). In contrast, there were no significant differences in mRNA levels for *Foxo1*, *Igf1bp2*, *Irs2*, *PBEF1/visfatin*, *Pgc1a*, *Sirt1*, *Nox4*, *Sod2*, *Cyp2d22*, *Gnmt*, *Cdkn2a*, *Mat1a*, *Hspb1*, or *Nrf1* (data not shown).

We then compared gene expression in the livers of female *Irs1*^{-/-} and WT mice at 80 and 450 days of age using whole genome oligonucleotide arrays. Expression values, biologically annotated using GO and Interpro, were analyzed using Catmap (36) to identify alter-

ations, at the level of biochemical and cellular processes, between the two genotypes at both ages. The majority of the common up-regulated gene classes were associated with energy metabolism including GO categories carbohydrate metabolism, oxidative phosphorylation, and tricarboxylic acid cycle (Table 1). The Interpro classes flavin-containing monooxygenases 1 and 5 were up-regulated (Table 1). GO categories down-regulated in 80- and 450-day-old female *Irs1*^{-/-} mice included immune response and the related antigen processing and serine esterase activity (Table 1).

DISCUSSION

Our findings provide new insights into the specific intracellular signaling pathways that mediate the effects of IIS on life span in mammals. Our data suggest that deletion of *Irs1* but not *Irs2* extends life span in female mice. Taken together with data from other long-lived mouse models, these findings are consistent with longevity control *via* an endocrine-signaling axis involving

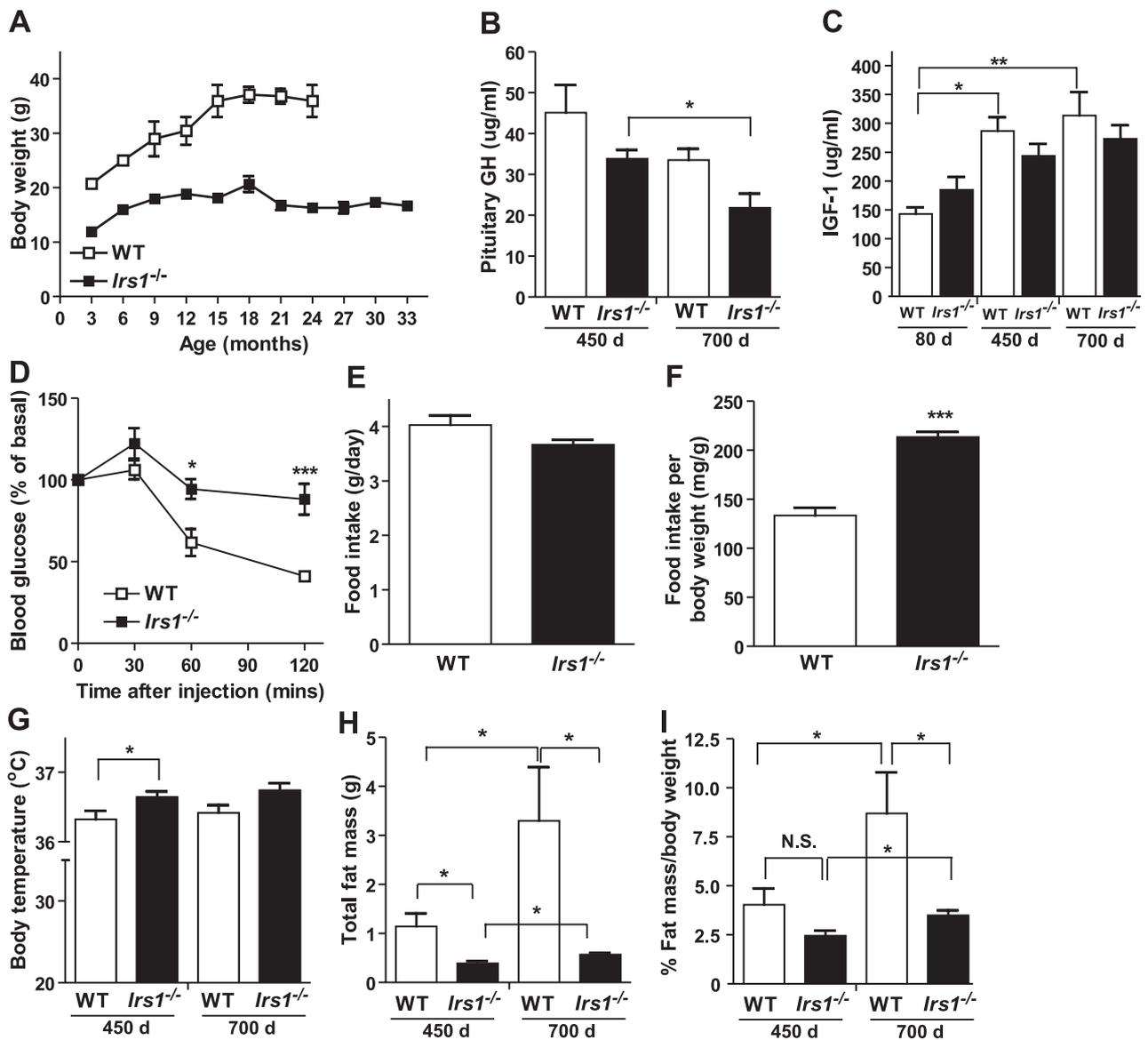


Figure 4. Female *Irs1*^{-/-} mice have reduced body weight but have normal pituitary endocrine function and are not calorically restricted. **A)** Growth curves in female *Irs1*^{-/-} and WT mice ($n=12$ per genotype; $P<0.01$ at all data points between WT and *Irs1*^{-/-} mice). **B)** GH levels at 450 and 700 days of age in female WT and *Irs1*^{-/-} mice ($n=4-6$ per genotype). **C)** Serum total IGF1 levels at 450 and 700 days of age in female WT and *Irs1*^{-/-} mice ($n=4-6$ per genotype). **D)** IGF1 tolerance tests in 450-day-old female WT and *Irs1*^{-/-} mice ($n=6$ per genotype). **E)** Food intake in 450-day-old female WT and *Irs1*^{-/-} mice ($n=12$ per genotype). **F)** Food intake per gram of body weight in 450-day-old WT and *Irs1*^{-/-} mice ($n=12$ per genotype). **G)** Rectal core body temperature in 450 and 700-day-old female WT and *Irs1*^{-/-} mice ($n=5-8$ per genotype). **H)** Total fat mass at 450 and 700 days of age in female WT and *Irs1*^{-/-} mice ($n=4-6$ per genotype). **I)** Fat mass per gram of body weight in 450- and 700-day-old female WT and *Irs1*^{-/-} mice ($n=4-6$ per genotype). For all panels results are means \pm SE. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; N.S., not significant.

IGF1, IGF1R, and IRS1. Male and female *Irs2*^{-/-} mice are short-lived because of their diabetic phenotype and neither mice heterozygote for *Irs1*, consistent with the preliminary observations of others (44), nor for *Irs2* show increased life span. Thus, IRS1 and IRS2 are likely to be differentially coupled to upstream and downstream signaling elements with IRS1 acting in different tissues and cell types and on different downstream regulated genes to control longevity.

Our studies also show for the first time that specific manipulation of IIS signaling components results in resistance to a number of age-related biomarkers. Female *Irs1*^{-/-} mice displayed better motor control, coordination, and balance than wild-type mice at 450 days of age, as demonstrated by improved performance in the Rotarod test. Aging also leads to a progressive deterioration in immune function in both mice and humans (45). In humans, immune senescence poten-

TABLE 1. Process-level conservation of hepatic transcriptional responses between 80- and 450-day-old female *Irs1*^{-/-} mice compared with age-matched WT controls

Category	Catmap P value	
	80-day <i>Irs1</i> ^{-/-}	450-day <i>Irs1</i> ^{-/-}
Up-regulated GO categories		
Biological process		
GO:0006091 Generation of precursor metabolites and energy	***	*
GO:0006092 Main pathways of carbohydrate metabolic process	*	*
GO:0045333 Cellular respiration	***	**
GO:0009060 Aerobic respiration	***	*
GO:0006119 Oxidative phosphorylation	*	**
GO:0042773 ATP synthesis coupled electron transport	*	***
GO:0046356 Acetyl CoA catabolic process	***	*
GO:0006099 Tricarboxylic acid cycle	***	*
GO:0009109 Coenzyme catabolic process	***	**
GO:0051187 Cofactor catabolic process	**	**
GO:0006084 Acetyl CoA metabolic process	**	*
Cellular component		
GO:0031975 Envelope	*	*
GO:0044422 Organelle part	***	*
GO:0005739 Mitochondrion	**	*
GO:0044429 Mitochondrial part	***	**
GO:0005740 Mitochondrial envelope	**	**
GO:0005743 Mitochondrial inner membrane	**	**
GO:0019866 Organelle inner membrane	**	*
GO:0031966 Mitochondrial membrane	**	*
GO:0005746 Mitochondrial electron transport chain	*	***
GO:0044455 Mitochondrial membrane part	*	***
GO:0031967 Organelle envelope	*	*
GO:0005747 Respiratory chain complex I	*	*
GO:0045261 Proton transporting ATP synthase complex catalytic core F 1	*	*
Molecular function		
GO:0016655 Oxidoreductase activity acting on NADH or NADPH quinone	*	***
GO:0003954 NADH dehydrogenase activity	*	***
GO:0004499 Dimethylaniline monooxygenase N oxide forming activity	**	**
GO:0015077 Monovalent inorganic cation transporter activity	*	***
GO:0015078 Hydrogen ion transporter activity	*	***
Up-regulated Interpro categories		
IPR002253 Flavin-containing monooxygenase 1	**	**
IPR002257 Flavin-containing monooxygenase 5	**	**
IPR000694 Proline rich region	*	***
Down-regulated GO categories		
Biological process		
GO:0006955 Immune response	***	*
GO:0002495 Antigen processing and presentation of peptide antigen via MHC class II	**	*
Cellular component		
GO:0045211 Postsynaptic membrane	*	*
Molecular function		
GO:0019904 Protein domain specific binding	**	*
GO:0004091 Carboxylesterase activity	*	**
GO:0004759 Serine esterase activity	*	**

Significantly up- or down-regulated functional categories (GO and Interpro) with significance determined using Catmap. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$. MHC = major histocompatibility complex.

tially leads to an increased risk of infection in the elderly population (45). T cell subset analysis in female *Irs1*^{-/-} mice revealed a reduced proportion of memory cells and increased numbers of naive cells compared to those in aged control animals. These results also support the view that T cell subsets can provide robust biomarkers of aging (38). In addition, female *Irs1*^{-/-} mice were completely resistant to age-related dermatitis, a condition that has been shown to affect C57BL/6

mice and is thought to be due to inflammation (37). Thus, it is possible that resistance to age-related increases in inflammatory processes protects against aging in *Irs1*^{-/-} mice as age-related increases in inflammation appear to contribute to human aging (46). Bone loss with advancing age is a major underlying cause of osteoporotic fractures in elderly humans. Female *Irs1*^{-/-} mice were more resistant than control animals to the age-related decline in bone mass despite

reports that IRS1 is required for normal bone growth in young mice (41). Therefore, female *Irs1*^{-/-} mice were resistant to a number of age-related changes associated with neurological and neuromuscular, immune, skin, and bone diseases.

Our studies also give insights into the potential mechanisms by which alteration of IIS regulates longevity. Although *Irs1*^{-/-} mice displayed reduced adiposity, which is also a feature of CR and FIRKO mice, our findings show that increased longevity can be uncoupled from improvements in insulin sensitivity associated with reduced fat mass. Therefore, although *Irs1*^{-/-} mice, like CR and FIRKO mice, did not develop an age-related deterioration in glucose homeostasis, in these animals this lack of deterioration was due to β -cell compensation in the face of lifelong insulin resistance rather than increased insulin sensitivity. Consistent with these findings, long-lived *C. elegans* and *Drosophila* IIS mutants and pituitary dwarf mice accumulate fat as they age, although in the latter case, this accumulation may reflect reduced lipolytic effects of GH and/or increased insulin sensitivity (2). Moreover, if leptin-deficient *ob*^{-/-} mutant mice are subjected to CR, they remain fat (48% body fat) yet show the same extension of life span as *ob*^{+/+} mice subjected to CR (14% body fat) (47). These observations suggest that, rather than absolute fat mass, alterations in the endocrine function of fat may be key to the regulation of longevity. This hypothesis is also supported by the findings that enhanced FOXO function in adipose tissue in *C. elegans* and *Drosophila* increases longevity (2). The beneficial effects of short-term CR on metabolic parameters and muscle glucose metabolism are, in fact, preserved in *Irs1*^{-/-} mice, further suggesting dissociation between the effects of CR and *Irs1* deletion (48). An additional observation of relevance to the interaction between insulin resistance and extended life span is the finding that overexpression of the putative hormone *Klotho* in mice extends life span and concomitantly impairs insulin action (44). In contrast, deletion of *Klotho* results in a phenotype suggestive of accelerated aging (49). However, although potentially providing further evidence that life span can be extended in the face of insulin resistance, the situation is complicated by the findings that *Klotho* also has significant interactions with FGF23 and calcium metabolism (50). Mice lacking FGF23 display an accelerated aging phenotype and hypoglycemia, and, therefore, current evidence suggests that *Klotho* is unlikely to extend life span purely by inhibiting insulin action (50).

Female *Irs1*^{-/-} mice have a reduced body size, but, unlike the long-lived pituitary hormone-deficient dwarf strains and GH receptor null mice, pituitary hormone levels are preserved. In addition, GH and thyroxine replacement in *Pit1*^{dww} mice increases body size and induces puberty while preserving the long-lived phenotype (51). These findings suggest that the thyroxine, prolactin, and GH deficiencies seen in *Prop1*^{df}, *Pit1*^{dww},

and *Ghrhr*^{lit} dwarf strains may not be the sole contributor to life span extension. However, although direct comparisons remain to be formally undertaken, the absolute life span extension seen in *Prop1*^{df}, *Pit1*^{dww}, and *Ghrhr*^{lit} dwarf strains is greater than that seen in the pure IIS mutants, suggesting that IRS1-independent but GH/thyroxine-dependent effects may play a distinct role in regulating longevity. Female *Irs1*^{-/-} mice also had normal RMR and a mildly elevated core temperature at 450 days of age and were not calorie restricted. Together these findings suggest that reduced food intake and altered metabolic parameters are not required for life span extension in female *Irs1*^{-/-} mice. In addition, the observed life span extension was apparent only in female but not in male *Irs1*^{-/-} mice. This result is consistent with the findings that in *dlnR* and *chico* mutant flies and in *Igf1r*^{+/-} mice life span extension is predominantly seen in female animals (2, 4–6). The basis of this sexually dimorphic phenotype is not clear, but it does provide further evidence that the mechanisms underlying life span extension in IIS mutants do not completely overlap with those of the pituitary dwarf mice and mice under CR, for which life span is increased in both sexes. However, because of the smaller numbers of male *Irs1*^{-/-} mice studied, we cannot formally exclude a modest increase in life span in these animals, and, indeed, we detected alterations in two age-sensitive markers of aging in these animals.

To further understand the mechanisms of life span extension in female *Irs1*^{-/-} mice we undertook both candidate gene expression analysis and whole genome microarray studies on livers from female *Irs1*^{-/-} and WT animals. Such data are currently available for some of the pituitary dwarf strains but not for *Igf1r*^{+/-} and FIRKO mice. Young *Irs1*^{-/-} mice show increased expression of the glutathione S-transferase *Gsta4*, which detoxifies the lipid peroxidation product 4-hydroxynonenal (4HNE). Glutathione S-transferase isoforms active against 4HNE are also up-regulated in long-lived *daf-2* mutant worms, *chico*^{+/-} flies, and *Prop1*^{df} and *Ghrhr*^{lit} mice (52, 53; unpublished data). Catalase expression was also modestly increased, consistent with observations in *Prop1*^{df} mice (54). Thus, elements of antioxidant and detoxification defense are up-regulated in *Irs1*^{-/-} mice. *Ercc8* expression was increased at all ages in female *Irs1*^{-/-} mice. Mutations in *Ercc8* cause Cockayne syndrome type A, a segmental progeroid syndrome in which patients develop signs of aging and age-related pathological changes at an early age (55). Short-lived mice with compound mutations in the Cockayne syndrome type B gene and *Xpd* (DNA excision repair protein ERCC-2) paradoxically show suppression of the somatotrophic axis, suggesting a complex interplay between IIS and DNA repair mechanisms (56).

Our analysis of liver microarrays encompassed identification of cellular and biochemical processes differentially expressed between female *Irs1*^{-/-} and control mice at both 80 and 450 days of age. This

approach demonstrated increased expression of genes involved in energy metabolism and, in particular, mitochondrial genes across a number of categories. Similar changes have been found in the livers of long-lived *Prop1^{df}* and *Ghrhr^{lit}* mice and during acute and long-term CR (53, 57, 58), suggesting that altered mitochondrial metabolism may be important during life span extension. Mice selected for increased metabolic efficiency have been reported to be long-lived (59). Immune response, antigen processing, and serine esterase categories were reduced in female *Irs1^{-/-}* mice, consistent with the immune changes seen in these animals and the evidence for reduced inflammation. Down-regulation in humoral defense and inflammatory response-related transcripts have been described previously in the livers of Ames and Little mice (53) and during long-term CR (60). These gene expression results suggest some commonality in the underlying mechanisms in female *Irs1^{-/-}* mice and other long-lived mouse models.

In conclusion, our findings provisionally suggest that a reduction in IRS1-dependent signaling is an evolutionarily conserved mechanism of mammalian life span regulation. Critically, the observed increase in life span in female *Irs1^{-/-}* mice is accompanied by maintenance of youthful characteristics and a reduction in age-sensitive markers of aging. Taken together with the data from lower organisms and other mouse models, IRS1 and the processes that it regulates may be points of intervention for therapies with the potential to delay age-related processes in humans. FJ

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