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Science **299**, 572 (2003);
DOI: 10.1126/science.1078223

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

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54. We thank S. Ghaznavi and H. J. Alitto for their assistance with the animals, and L. Ungerleider [National Institute of Mental Health (NIMH) and NIH] and J. Rauschecker (Georgetown University) for their critical reading of the manuscript and helpful suggestions. Supported by NIMH-IRP, NIH, and the U.S. Department of Health and Human Services.

Supporting Online Material
www.sciencemag.org/cgi/content/full/299/5606/568/DC1
Materials and Methods
Fig. S1

30 September 2002; accepted 4 December 2002

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Caloric restriction has been shown to increase longevity in organisms ranging from yeast to mammals. In some organisms, this has been associated with a decreased fat mass and alterations in insulin/insulin-like growth factor 1 (IGF-1) pathways. To further explore these associations with enhanced longevity, we studied mice with a fat-specific insulin receptor knockout (FIRKO). These animals have reduced fat mass and are protected against age-related obesity and its subsequent metabolic abnormalities, although their food intake is normal. Both male and female FIRKO mice were found to have an increase in mean life-span of ~134 days (18%), with parallel increases in median and maximum life-spans. Thus, a reduction of fat mass without caloric restriction can be associated with increased longevity in mice, possibly through effects on insulin signaling.

Longevity is dependent on many factors including genetics (*1*, *2*), hormonal and growth factor signaling (*3*, *4*), body weight (*5*), body fat content, and environmental factors (*4*, *6*). Food restriction is the most potent environmental variable and has been shown to increase longevity in diverse organisms (*6*). The effect of restricted feeding on life-span has been studied in rodents for more than 60 years (*7*–*10*), and although some studies have suggested that reduced food intake is more important than adiposity (*8*, *9*), it is difficult to separate the beneficial effect of caloric restriction from that of leanness and the various biochemical correlates of leanness.

To investigate this question, we evaluated the life-span of the fat-specific insulin receptor knockout (FIRKO) mouse. These animals were derived by crossing insulin receptor IR (lox/+) mice, in which exon 4 of the insulin receptor is flanked by loxP sites (*11*), with IR (lox/+) mice that also express the Cre recombinase under the control of the aP2 promoter/enhancer (*12*). This breeding strategy also generated three littermate control groups: wild-type (WT), IR (lox/lox), and aP2-Cre mice, which were indistinguishable with regard to physiologic and metabolic parameters and have the same mixed genetic background as the FIRKO mice. For the aging experiments, 250 animals were housed under the same conditions in a virus-free facility on a 12-hour light/dark cycle and were given a standard rodent feed and water ad libitum.

Growth curves were normal in male and female FIRKO mice from birth to 8 weeks of age. Starting at 3 months of age, FIRKO mice maintained 15 to 25% lower body weights and a 50 to 70% reduction in fat mass throughout life (Fig. 1A). The reduction in adiposity was estimated by perigonadal fat pad weight but was apparent in all fat depots and was also reflected by a reduction of ~25% in total-body triglyceride content (*13*). FIRKO mice are healthy, lack any of the metabolic abnormalities associated with lipodystrophy, and are protected against age-related deterioration in glucose tolerance, which is observed in all control strains (*13*). FIRKO mice maintained low body fat, despite normal food intake (Fig. 1B). Indeed, because FIRKO mice were leaner, the food intake of FIRKO mice expressed per gram of body weight actually exceeded that of controls by an average of 55% (Fig. 1C).

The median life-span of most laboratory

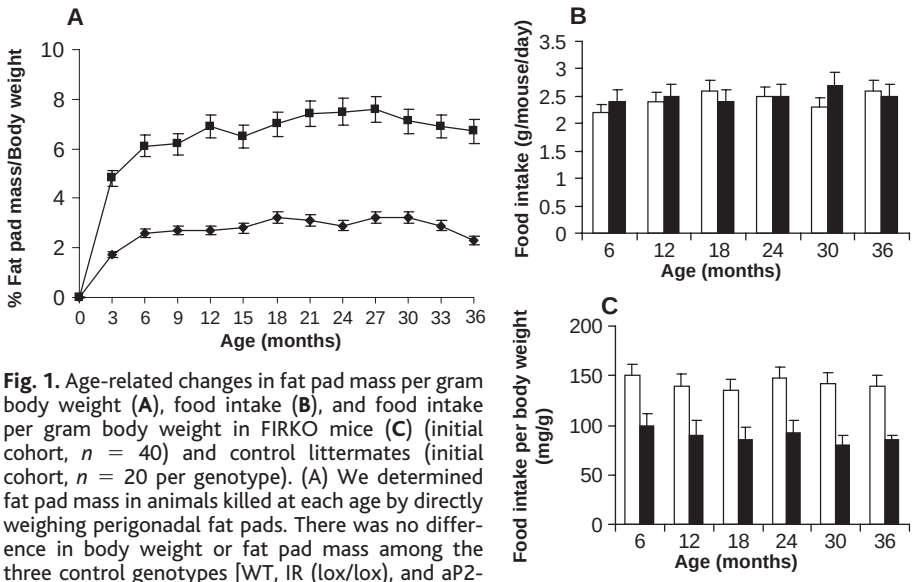

Fig. 1. Age-related changes in fat pad mass per gram body weight (**A**), food intake (**B**), and food intake per gram body weight in FIRKO mice (**C**) (initial cohort, $n = 40$) and control littermates (initial cohort, $n = 20$ per genotype). (A) We determined fat pad mass in animals killed at each age by directly weighing perigonadal fat pads. There was no difference in body weight or fat pad mass among the three control genotypes [WT, IR (lox/lox), and aP2-Cre]. After the mice reached the age of 3 months, the differences in fat pad mass per gram of body weight were significant for all data points between FIRKO mice (diamonds) and all three controls (squares) ($P < 0.05$). (B) In mice caged singly, we determined food intake (gram per mouse per day) daily over 5 days by using at least five FIRKO (white bars) and four control mice (black bars) per genotype ($n = 12$). Data of the control genotypes [WT, IR (lox/lox), and aP2-Cre] are plotted together in the black bars, because there were no differences in daily food intake among them. (**C**) Food intake per gram body weight—calculated from the food intake and body weight data—was significantly increased in FIRKO mice (white bars) as compared with controls (black bars) ($P < 0.05$).

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mice, such as BALB/c-nu (*5*) or C57BL/6J (*14*), is 30 months. Consistent with this, we found that 45 to 54% of the mice in the control groups lived to 30 months of age. By contrast, about 80% of the FIRKO mice were alive at this age (Fig. 2A). The increased survival of FIRKO mice was seen in both males and females and was confirmed in two independent lines of mice. Complete survival curves revealed that the mean life-span was increased by 134 days (from 753 to 887) or 18% (Fig. 2B). The median life-span in FIRKO mice was also increased by 3.5 months (from 30 ± 0.6 months to 33.5 months), and the maximum life-span was extended by ~5 months. At 36 months, all mice in the control groups had died, whereas ~25% of the FIRKO mice were still alive. The longest lived FIRKO mice died at the age of 41 months. Further analysis of the survival plots (Fig. 2B) with previously described mathematical models (*15*) revealed that extended longevity in FIRKO mice is associated with both a shift in the age at which the "age-dependent increase in mortality risk" becomes appreciable and a decreased rate of age-related mortality, especially after 36 months of age.

The FIRKO mouse demonstrates the beneficial effects of reduced adiposity on the extension of life-span in a setting where food intake is normal or even increased relative to body weight. Caloric restriction has been hypothesized to delay aging by decreasing metabolism and the associated production of damaging oxygen free radicals (*16*–*19*). In the nematode *Caenorhabditis elegans* (*20*) and the fruit fly *Drosophila melanogaster* (*21*), slowing down mitochondrial metabolism and metabolic rate appears to extend life expectancy. However, in rodents, caloric restriction appears to extend life-span without decreasing the metabolic rate (*22*, *23*). In FIRKO mice, the resistance to obesity, despite normal food intake, suggests that metabolic rate is increased, rather than decreased (*13*). If free radical damage is the important factor, then in the FIRKO mouse this must be derived directly or indirectly from the decreased fat mass rather than the diet. Another possibility is that the increased longevity in FIRKO mice is the direct result of altered insulin signaling. Mutations that reduce signaling through the insulin-like signaling pathway can increase life expectancy in *C. elegans* (*24*–*26*) and in *Drosophila* (*27*, *28*), and these longevity mutations can be reversed in some cases by additional activating mutations in the insulin/IGF-1 signaling pathway. In both of these species, reduced insulin-like signaling can extend life-span by 50% or more (*20*, *27*, *28*).

Although decreased insulin-like signaling appears to increase life expectancy in invertebrates, whether the same is true in mammals or humans is unclear. At least three genes have been identified (*Pit1*^{dw}, *Prop1*^{df}, *Ghr*) in which loss-of-function mutations lead to dwarfism with reduced levels of IGF-1 and insulin. These are associated with increased longevity in mice (*3*, *4*). On the other hand, loss-of-function mutations in the insulin receptor, which lead to severe insulin resistance (*29*–*31*), or even milder forms of insulin resistance associated with diabetes and obesity (*32*, *33*), result in shortened life-span in both humans and mice. FIRKO mice have a selective loss of insulin signaling in adipose tissue only, and this is not associated with diabetes or glucose intolerance. Moreover, FIRKO mice have normal to somewhat supernormal glucose tolerance and, thus, may in some ways mimic caloric restriction, which is known to extend life-span in rodents (*10*). Indeed, the phenotype of aging FIRKO mice shows similarities with the phenotype of food-restricted mice, such as reduced adiposity, trend to lower insulin levels, and protection from decreased insulin sensitivity (*13*).

In summary, the results of our studies with FIRKO mice are consistent with the view that leanness, not food restriction, is a key contributor to extended longevity. The exact mechanism underlying this effect requires further analysis.

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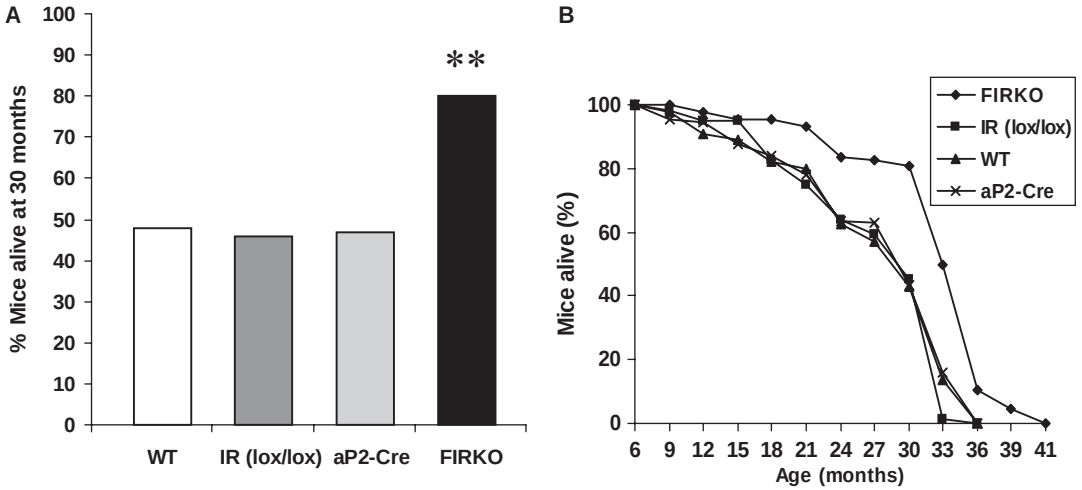

Fig. 2. Extended life-span in FIRKO mice. (**A**) Percentage of mice alive at 30 months of age. The median life-span of the three control genotypes [WT, IR (lox/lox), aP2-Cre] was ~30 months. For this analysis, the fraction of mice alive at 30 months from founder line 1 [WT (n = 34), IR (lox/lox) (n = 35), aP2-Cre (n = 30), and FIRKO (n = 32)] and from line 2 [WT (n = 33), IR (lox/lox) (n = 31), aP2-Cre (n = 27), and FIRKO (n = 28)] were pooled. Data for males and females were also pooled, because they were similar in each of the groups. **P < 0.05. (**B**) Pooled survival curves for FIRKO mice derived from two different aP2-Cre founder lines. We performed pair-wise comparisons among genotypes for age-specific survival by log-rank test with significance corrected for multiple tests. Median life-span in line 1 (n = 131) was FIRKO, 33.5 months; WT, 30.3 months; IR (lox/lox), 28.7 months; and aP2-Cre, 30.7 months. Among the control groups WT, IR (lox/lox), and aP2-Cre, survival did not differ (P = 0.31), whereas survival of FIRKO mice was significantly increased (P < 0.001). Median life-span in line 2 (n = 119) was FIRKO, 33.4 months; WT, 29.8 months; IR (lox/lox), 30.1 months; and aP2-Cre, 29.9 months. The maximum longevity (average life-span of the 10% longest lived mice) was significantly increased from 34.7 months in the controls to 39.5 months in FIRKO mice (P < 0.001). Among the control groups WT, IR (lox/lox), and aP2-Cre, maximum life-span did not differ (P = 0.62). The curves shown represent the pooled data from both of these lines.

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9 September 2002; accepted 11 December 2002

Disruption of Transforming Growth Factor–β Signaling in ELF β-Spectrin–Deficient Mice

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Disruption of the adaptor protein ELF, a β-spectrin, leads to disruption of transforming growth factor–β (TGF-β) signaling by Smad proteins in mice. *Elf* $^{-/-}$ mice exhibit a phenotype similar to *smad2*$^{+/-}$/*smad3*$^{+/-}$ mutant mice of midgestational death due to gastrointestinal, liver, neural, and heart defects. We show that TGF-β triggers phosphorylation and association of ELF with Smad3 and Smad4, followed by nuclear translocation. ELF deficiency results in mislocalization of Smad3 and Smad4 and loss of the TGF-β–dependent transcriptional response, which could be rescued by overexpression of the COOH-terminal region of ELF. This study reveals an unexpected molecular link between a major dynamic scaffolding protein and a key signaling pathway.

Transforming growth factor–β (TGF-β) signals are conveyed through serine-threonine kinase receptors at the cell surface to specific intracellular mediators, the Smad proteins (*1*). Activation of Smad proteins results in their translocation to the nucleus and subsequent activation of gene expression (*2*). Vertebrates possess at least nine Smad proteins (*3–8*), which fall into three functional classes: (i) receptor-activated Smads (R-Smads)—Smad1, Smad2, Smad3, Smad5, and Smad8; (ii) co-mediator Smads—Smad4 and Smad10; and (iii) inhibitory Smads—Smad6 and Smad7. Activity of R-Smads and Smad4 can be modulated by adaptor proteins in the cytosol such as filamin and Smad anchor for receptor activation (SARA). Because such adaptors can control Smad access to TGF-β receptors (TβRI and TβRII), which activate Smad at the cell surface membrane, they play a critical role in facilitating TGF-β functions such as growth, differentiation, vascular remodeling, and cell fate specification (*1–9*).

Cytoskeletal proteins belonging to the β-spectrin family are thought to regulate signal transduction by functioning as adaptor molecules (*10–12*). When expression of the β-spectrin gene *elf* (embryonic liver fodrin) was blocked, liver formation was inhibited (*13*), and a phenotype similar to mice with compound haploinsufficiency at Smad2 and Smad3 loci was seen (*14, 15*). To assess a possible role in TGF-β signaling, we generated ELF-deficient mice by gene targeting (fig. S1, Fig. 1) (*16*). Homozygous mutant *elf*$^{-/-}$ mice were not detected, indicating that the *elf* mutation is a recessive embryonic lethal. Abnormal or degenerating embryos were recovered between embryonic day 8.5 (E8.5) and E16.5. At E9.5, *elf*$^{-/-}$ embryos were readily distinguished from their wild-type littermates by their smaller body and head size and lack of a branching network of vessels in the yolk sac (Fig. 1B). *Elf*$^{-/-}$ embryos were severely distorted at E11.5, with growth retardation and multiple defects (Fig. 1, C to G). Cardiovascular defects included an absence of the normal trabeculated pattern of myocardial tissue with altered, thickened myocardial fibers. The myoblasts in the *elf*$^{-/-}$ mutants were markedly hyperplastic with an absence of linear arrangement of nuclei, resulting in a small ventricular lumen, and occlusion at the atrioventricular region (Fig. 1, E and F). Phenotypic similarities between *smad2*$^{+/-}$/*smad3*$^{+/-}$ and *elf*$^{-/-}$ embryos included abnormal anatomy of primary brain vesicles (Fig. 1D), craniofacial abnormalities, aberrant gut formation, severe hypoplasia of the liver, and distorted liver architecture. In the *elf*$^{-/-}$ liver, hepatocytes were not always arranged in cords, and there were few early intrahepatic bile ducts (Fig. 1, E and I). Reduced expression of α-fetoprotein, a liver marker, in *elf*$^{-/-}$ and *smad2*$^{+/-}$/*smad3*$^{+/-}$ mutants indicates that, although hepatic lineage is established, further differentiation and growth may be arrested (Fig. 1I).

The phenotypic similarity between *smad2*$^{+/-}$/*smad3*$^{+/-}$ and *elf*$^{-/-}$ mutants suggested cross talk between ELF and the Smad gene family. Yolk sac blood vessel dilatation observed in some of the *elf*$^{-/-}$ mutants is reminiscent of the TβRI, TβRII, activin receptor-like kinase-1 (ALK1), and Smad5 mutants, suggesting a role for ELF in TGF-β signaling (*3, 17*). Analysis of mouse embryonic fibroblasts (MEFs) derived from wild-type and *elf*$^{-/-}$ mutants showed that the *elf*$^{-/-}$ MEFs did not respond to TGF-β1 stimulation, but they did respond to platelet-derived growth factor (PDGF) (Fig. 1H) (*18, 19*). This was confirmed by transient transfection experiments in which reporter constructs containing Smad binding sequences upstream of a luciferase gene were expressed in wild-type and *elf*$^{-/-}$ MEFs (*19, 20*).

To determine whether ELF associates with Smad2, Smad3, and Smad4, we immunoprecipitated endogenous ELF from cell extracts prepared from wild-type MEFs and HepG2 (a human liver cell line) cells that had been treated with TGF-β1 (*16*, *21*). TGF-β1 treatment stimulated ELF and Smad3 phosphorylation (Fig. 2; fig. S2, A and B). In all cells, specific antisera to ELF immunoprecipitated Smad3 and Smad4 proteins only in the presence of TGF-β1 (Fig. 2; fig. S2C). No association occurred with Smad2 (Fig. 2; fig. S2C). In contrast, ELF associated with two known spectrin binding structural proteins, ankyrin B and tropomyosin, only in the absence of TGF-β1 (Fig. 2). This suggests that, upon stim-

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