

SIRT1 transgenic mice show phenotypes resembling calorie restriction

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Summary

We generated mice that overexpress the sirtuin, SIRT1. Transgenic mice have been generated by knocking in SIRT1 cDNA into the β -actin locus. Mice that are hemizygous for this transgene express normal levels of β -actin and higher levels of SIRT1 protein in several tissues. Transgenic mice display some phenotypes similar to mice on a calorie-restricted diet: they are leaner than littermate controls; are more metabolically active; display reductions in blood cholesterol, adipokines, insulin and fasted glucose; and are more glucose tolerant. Furthermore, transgenic mice perform better on a rotarod challenge and also show a delay in reproduction. Our findings suggest that increased expression of SIRT1 in mice elicits beneficial phenotypes that may be relevant to human health and longevity.

Key words: calorie restriction; SIRT1; transgenic mice.

Introduction

The Sir2 gene promotes longevity in yeast, *Caenorhabditis elegans* and *Drosophila* (Kaeberlein *et al.*, 1999; Tissenbaum & Guarente, 2001; Rogina & Helfand, 2004). Sir2 protein and its mammalian ortholog, SIRT1, are nicotinamide adenine dinucleotide (NAD)-dependent deacetylases that may function to connect

metabolism to lifespan (Bordone & Guarente, 2005). Indeed, at least some of the effects of moderate calorie restriction (CR) require Sir2-related genes in yeast, *Drosophila* and mice (Lin *et al.*, 2002, 2004; Wood *et al.*, 2004; Chen *et al.*, 2005).

Several links between SIRT1 and metabolic processes affected by CR have been reported, and the levels of SIRT1 are induced in several tissues in CR mice or rats (Cohen *et al.*, 2004). First, SIRT1 inhibits adipogenesis in white adipose tissue by repressing activity of the proadipogenic nuclear receptor, peroxisome proliferator-activated receptor γ (PPAR γ) (Picard *et al.*, 2004). Second, SIRT1 regulates insulin secretion by pancreatic β cells by regulating expression of the UCP2 uncoupling protein in those cells (Moynihan *et al.*, 2005; Bordone *et al.*, 2006). Third, SIRT1 deacetylates and activates the transcriptional coactivator PGC-1 α to drive expression of genes for gluconeogenesis in the liver (Rodgers *et al.*, 2005).

The putative small molecule activator of SIRT1, resveratrol, extends lifespan in yeast, *C. elegans* and *Drosophila* (Lamming, *et al.*, 2004; Wood *et al.*, 2004), and opposes effects of a high-fat diet in mice (Baur *et al.*, 2006; Lagouge *et al.*, 2006). More specifically, this compound increases metabolism and glucose tolerance in these mice and improves their physical performance in response to a rotarod challenge. Resveratrol may act by promoting deacetylation of PGC-1 α to activate mitochondrial gene expression and function in muscle and brown fat (Lagouge *et al.*, 2006).

Interestingly, SIRT1 $-/-$ mice are not viable in inbred strain backgrounds and show pleiotropic phenotypes in outcrossed strains, including small size, developmental defects and sterility (McBurney *et al.*, 2003). Because of the pleiotropy of the SIRT1 $-/-$ mice, it has been challenging to use these mice to study the effects of SIRT1 on physiology. Thus, we report here the construction and initial characterization of SIRT1 transgenic mice that express the protein from the SIRT1 cDNA knocked into the β -actin locus. Transgenic mice show increased SIRT1 protein levels in several tissues and display multiple physiological phenotypes that help to demonstrate the role of this sirtuin in mammalian physiology.

Results

Construction and characterization of SIRT1 transgenic mice

To generate SIRT1 transgenic mice, SIRT1 cDNA adjacent to loxP-Neo-loxP and an internal ribosome expression sequence (IRES) was knocked into the ubiquitously expressed β -actin locus (wild-type; WT) in embryonic stem cells to generate the preknockin allele, pre-KI (Fig. 1A). These targeted embryonic

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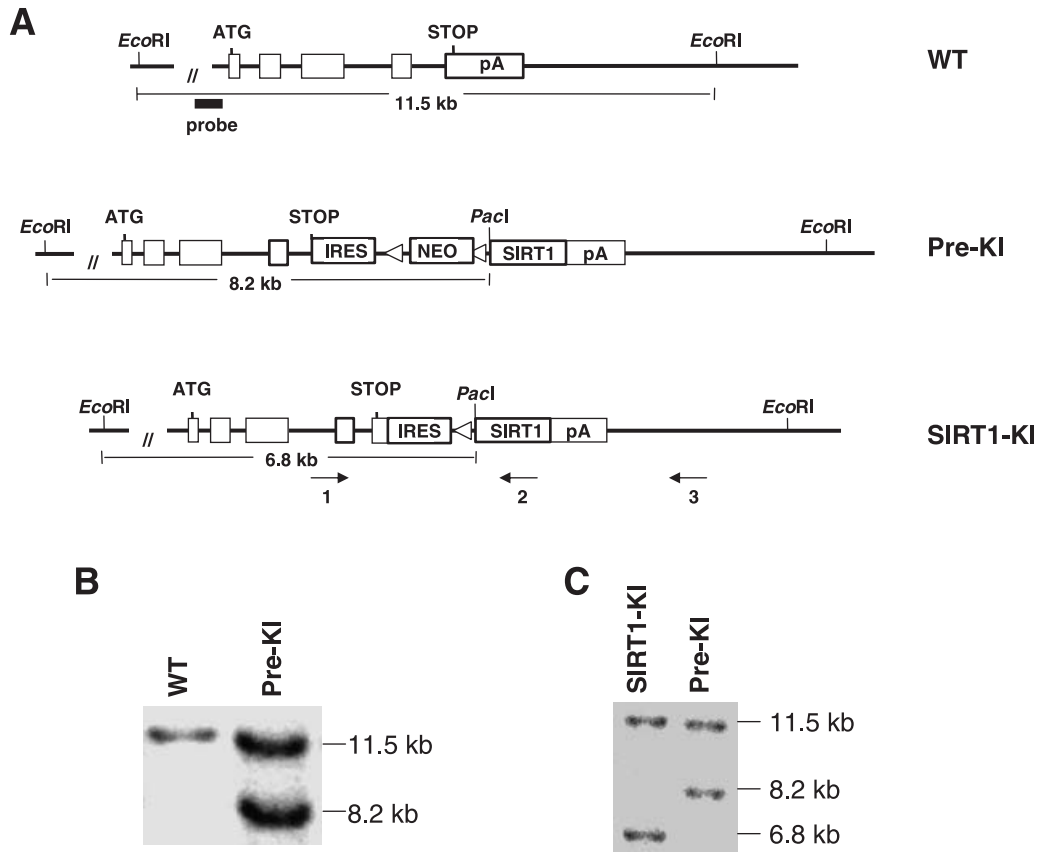


Fig. 1 Generation of SIRT1 transgenic mice. (A) The top diagram shows the genomic organization of the mouse β -actin locus (wild-type; WT), the middle diagram is the preknockin allele in embryonic stem clones (pre-KI) and the bottom diagram is the SIRT1 expressing allele in mice after removal of neo by Cre recombinase (SIRT1-KI). Also shown is the location of three primers used in genotyping. (B) Southern blot analysis of targeted embryonic stem cells using the 5' external probe shown in (A). Genomic DNA from embryonic stem clones was digested by *EcoRI* and *PacI*. The size of the WT band is 11.5 kb and of the pre-KI band is 8.2 kb. (C) Southern blot confirmation of the deletion of neomycin resistance gene by Cre recombinase. Mouse tail DNA was digested by *EcoRI* and *PacI* and probed with the probe in (A). The size of the WT band is 11.5 kb, the embryonic stem cell-targeted band is 8.2 kb, and the neo deleted band is 6.8 kb. SIRT1-KI: mouse with Cre-mediated recombination.

stem cells were injected into blastocysts to generate chimeras and germline transmission was achieved. The neo gene was then floxed out by crossing to Cre-expressing mice, placing the IRES in position to direct expression of the SIRT1 coding sequence fused to the β -actin mRNA (Fig. 1A). Targeting and Cre-mediated recombination were confirmed by Southern blotting (Fig. 1B,C). We term this knock-in allele SIRT1-KI. Note that the mRNA encoded by SIRT1-KI should also express normal β -actin as part of a bicistronic message.

In order to judge whether the transgene was expressed and functional, we crossed the SIRT1-KI mice to SIRT1 $-/-$ mice, as schematized in Fig. 2A. The SIRT1 $-/-$ mice without SIRT1-KI displayed phenotypes characteristic of this knockout strain (Cheng *et al.*, 2003; McBurney *et al.*, 2003), including small size and closed eye lids (Fig. 2B,C). Both of these phenotypes were suppressed in SIRT1 $-/-$ mice with SIRT1-KI (8/8 scored) compared to mice without the transgene (0/8 scored). Another phenotype of SIRT1 $-/-$ mice is sterility in both males and females, and this was also suppressed in mice with SIRT1-KI (3/3 males and 3/3 females) compared to mice without the transgene (0/3 males and 0/3 females). Expression of the SIRT1

protein from SIRT1-KI was observed by Western blotting lysates of white adipose tissue (WAT) of SIRT1 $-/-$ mice with the transgene (Fig. 2D). We conclude that SIRT1 is expressed from the SIRT1-KI allele and is functional in mice.

Next, SIRT1-KI was crossed into a SIRT1 $+/+$ background and littermates with and without the transgene were compared (see Experimental procedures for a description of this colony). We noted a significant under-representation of SIRT1-KI mice in the F_1 progeny of this colony (Supplementary Fig. S1). This deviation from the expected outcome was observed whether the transgene was passed from male or female parents, suggesting that the SIRT1-KI transgene may confer a selective disadvantage during embryonic development. Consistent with this idea, in a separate cross in which both parents were heterozygous for SIRT1-KI, mice homozygous for SIRT1-KI were never detected (in 68 F_1 progeny of heterozygous parents, $P < 0.005$).

SIRT1 mice overexpress the protein in WAT, brown adipose tissue (BAT), brain and mouse embryonic fibroblasts (MEF) (Fig. 3) and also in calvaria cells of the skull (not shown). However, overexpression was not apparent by Western blot in two other important metabolic tissues: liver and muscle (Supplementary

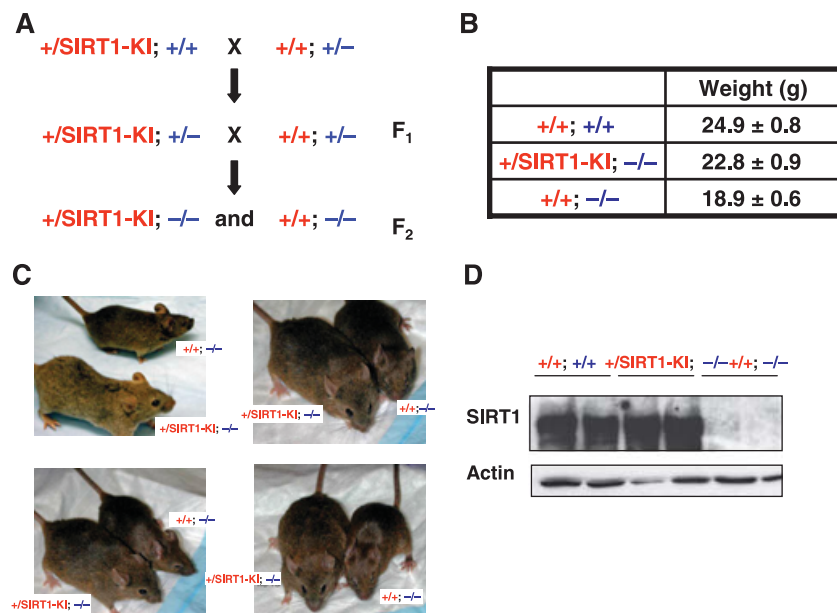


Fig. 2 The SIRT1-KI allele can rescue the SIRT1 knockout mice phenotypes and expresses SIRT1 protein. (A) Schematic representation of crossing strategy between mice carrying the SIRT1-KI allele ($+/\text{SIRT1-KI}; +/+$) and mice heterozygous at the SIRT1 locus ($+/+; +/-$). SIRT1 $+/+$ homozygotes and $+/-$ heterozygotes generated in the F₂ are not indicated. (B) Weight of males wild-type (WT) and males SIRT1 knockout mice ($n = 5$) with the indicated genotypes. (C) Photographs of typical littermates of SIRT1 knockout mice with the indicated genotypes. (D) Western blot for SIRT1 in mice of the indicated genotypes. White adipose tissues (WAT) of two mice (males) of each genotype are shown with actin used as a loading control.

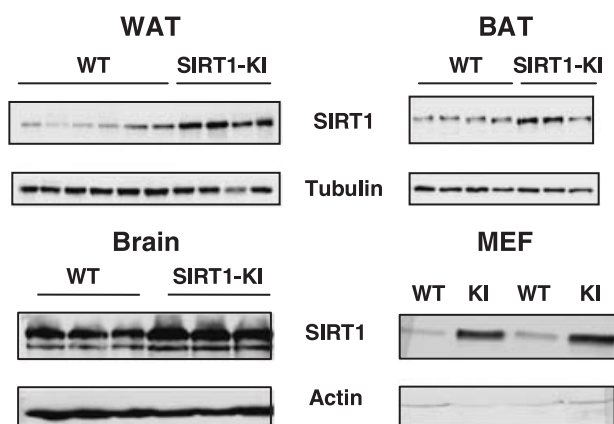


Fig. 3 Overexpression of SIRT1 protein SIRT1-KI mice. Mice with a wild-type (WT) SIRT1 locus and with or without the SIRT1-KI allele were generated as described in Experimental procedures. Western blot of SIRT1 in white adipose tissue (WAT), brown adipose tissue (BAT), brain and mouse embryonic fibroblasts (MEF) in WT and SIRT1-KI littermates. Each lane represents an individual mouse.

Fig. S2A). The β -actin-SIRT1 fusion mRNA was also evident after Northern blotting of RNA isolated from WAT (not shown). To ensure that transgenic mice expressed WT levels of β -actin protein from the SIRT1-KI allele, we compared β -actin protein levels in several tissues of transgenic mice compared to WT. Expression of β -actin protein was equivalent in SIRT1-KI and WT mice (Supplementary Fig. S2B), indicating that any phenotypes in transgenic animals must not result from an underproduction of this protein.

Physiological phenotypes of SIRT1-KI mice

We observed several phenotypes in SIRT1 $+/+$ mice that were hemizygous for the SIRT1-KI allele. First, the body weight of

SIRT1-KIs was lower than that of controls (Fig. 4A,B). The difference in body weights grew larger as the animals aged over a 10-month period. Because mice are known to gain WAT over this time, it seemed possible that this increasing weight difference was due to a lack of accumulation of fat in the SIRT1-KI mice. Indeed, measurements of visceral epididymal depots of WAT in 3-month-old animals showed a large reduction in fat mass in SIRT1-KI mice (Fig. 4C). In humans, this fat depot is associated with glucose intolerance and metabolic syndrome (Despres & Lemieux, 2006). The SIRT1-KI mice also displayed lower levels of circulating free fatty acids, leptin and adiponectin (Table 1). The reduction in leptin is consistent with the reduced fat mass in SIRT1-KI mice, while the reduction in adiponectin is consistent with the recent finding that SIRT1 represses adiponectin secretion (Qiang *et al.*, 2007). The relative mass of other tissues, such as gastrocnemius muscle, was indistinguishable in SIRT1-KI and WT mice (Fig. 4D).

Second, SIRT1-KI mice showed significantly lower levels of total blood cholesterol than controls (Table 1). This reduction was also reflected in individual measurements of both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Table 1). Because we failed to detect SIRT1 overexpression in liver, it is possible that these effects reflect altered cholesterol uptake in peripheral tissues in SIRT1-KI mice. Alternatively, the SIRT1 protein determination assay in liver may be a less sensitive assay than blood cholesterol assays. No differences were observed in levels of circulating triglycerides or glycerol in transgenic mice (Table 1), nor did we observe any significant changes in the circulating levels of the insulin-like growth factor (IGF), IGF-1, lactate, hydroxybutyrate or corticosterone in SIRT1-KI (Table 1).

Third, SIRT1-KI mice evinced improved glucose homeostasis. This difference was exemplified by a reduction in blood insulin levels in fed animals and in fasted glucose levels in 6-month-

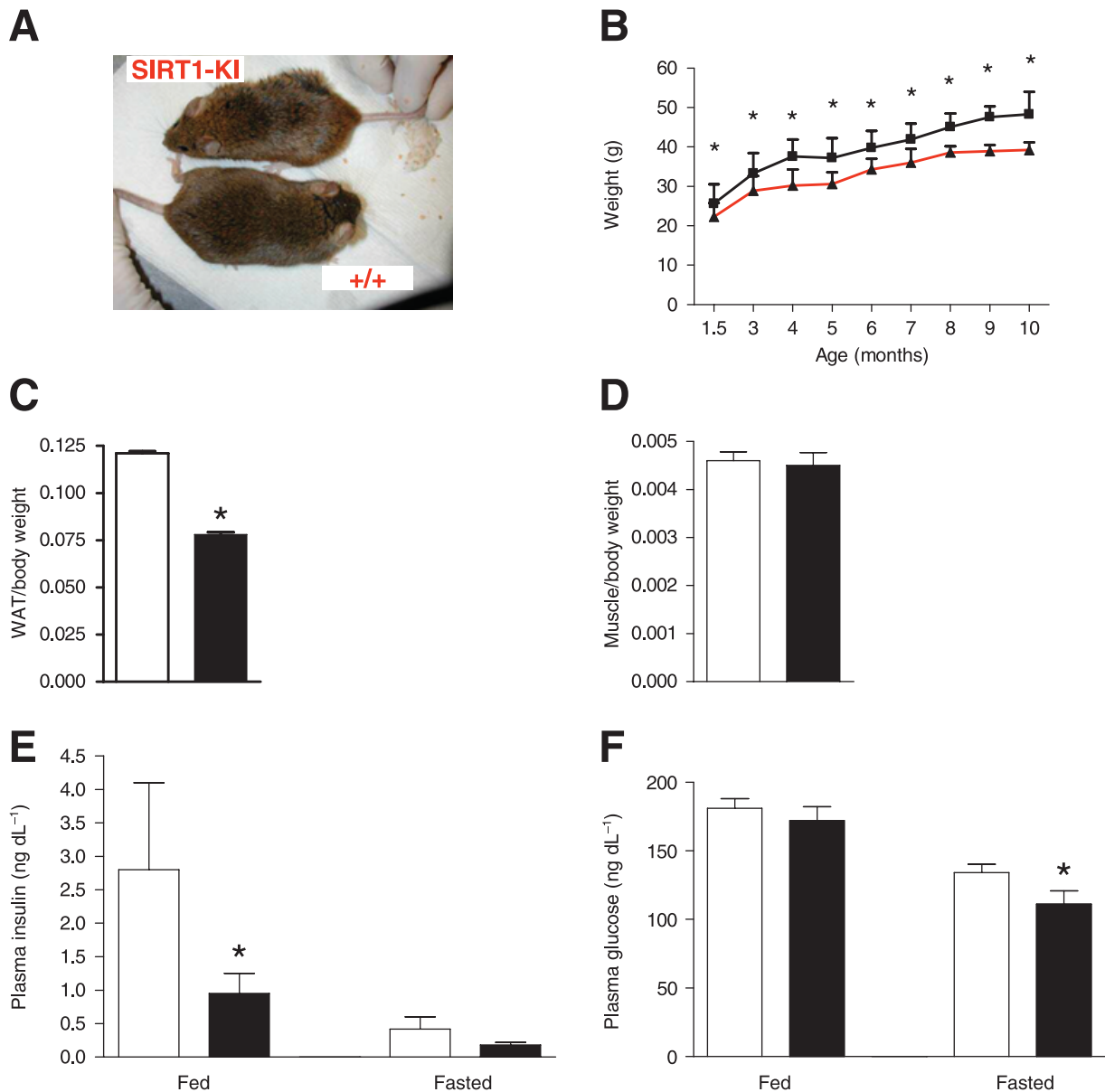


Fig. 4 Body weight, leptin and adiponectin in males SIRT1-KI mice. (A) Photograph of typical wild-type (WT) and SIRT1-KI littermates. (B) Body weight of WT (black line) and SIRT1-KI mice (red line). At all time-points the transgenic mice weigh significantly less than the WT mice ($*P < 0.05$, ANOVA; $n = 10$ –20 mice). (C) Mass of white adipose tissue (WAT) and (D) muscle of WT and SIRT1-KI mice ($n = 4$ –6) as percentage of body weight. $*P < 0.05$ (ANOVA). In this and all subsequent panels and figures, open bars are WT and black bars are SIRT1-KI mice. (E) Plasma insulin and (F) glucose levels were determined in blood of WT and SIRT1-KI mice ($n = 10$) at 6 months of age under fed or fasted conditions, as indicated. $*P < 0.05$ (ANOVA).

old mice (Fig. 4E,F). Similar results were observed in 1.5- and 3-month-old mice (data not shown). Moreover, SIRT1-KI mice showed an increase in glucose tolerance (the ability to clear a bolus of glucose from the blood) (Fig. 5A).

Fourth, SIRT1-KI mice showed an increase in metabolic rate. We observed a significant increase in food consumption normalized to body weight in transgenic mice (Fig. 5B), consistent with the possibility that the SIRT1-KI mice are more metabolically active than controls. Measurements of oxygen consumption provide further support for this idea by showing higher values

in the SIRT1-KI mice at 7 months of age compared to controls (Fig. 5C). A trend toward higher oxygen consumption was also noted in 3-month-old mice (not shown).

Organismal phenotypes of SIRT1-KI mice

The physiological phenotypes described above are changes also observed in WT mice during CR (Weindruch & Walford, 1988; Nisoli *et al.*, 2005; Baur *et al.*, 2006). In addition to longevity, two critical organismal hallmarks of CR in mice are improved

Table 1 Serum levels of different hormones, adipokines and fatty acids

		3 months	12 months
FFA (nmol L ⁻¹)	WT	1.8 ± 0.04 (n = 6)	1.99 ± 0.07 (n = 5)
	SIRT1-KI	1.74 ± 0.15 (n = 6)	1.62 ± 0.12* (n = 5)
Leptin (pg mL ⁻¹)	WT	0.46 ± 0.08 (n = 6)	0.86 ± 0.02 (n = 5)
	SIRT1-KI	0.28 ± 0.06* (n = 6)	0.53 ± 0.06* (n = 5)
Adiponectin (ngmL ⁻¹)	WT	14310 ± 373 (n = 6)	15320 ± 755 (n = 5)
	SIRT1-KI	12430 ± 785 (n = 6)	10250 ± 715* (n = 5)
Total cholesterol (mg dL ⁻¹)	WT	131 ± 10 (n = 6)	184 ± 9.6 (n = 5)
	SIRT1-KI	104 ± 4.6* (n = 6)	129 ± 12* (n = 5)
LDL (mg dL ⁻¹)	WT	10.8 ± 1.08 (n = 6)	16.3 ± 1.1 (n = 5)
	SIRT1-KI	11.75 ± 0.75 (n = 5)	11.2 ± 0.86* (n = 5)
HDL (mg dL ⁻¹)	WT	71.7 ± 5.9 (n = 6)	94.5 ± 8.8 (n = 4)
	SIRT1-KI	58.3 ± 5.4 (n = 5)	71.2 ± 8.0 (n = 5)
Triglycerides (mg dL ⁻¹)	WT	273 ± 35 (n = 6)	263 ± 9 (n = 5)
	SIRT1-KI	288 ± 37 (n = 6)	252 ± 45 (n = 5)
Glycerol (mg dL ⁻¹)	WT	7.05 ± 0.54 (n = 6)	8.05 ± 0.51 (n = 5)
	SIRT1-KI	6.4 ± 1.04 (n = 6)	7.04 ± 0.9 (n = 5)
Lactate (mg dL ⁻¹)	WT	41 ± 1.8 (n = 6)	ND
	SIRT1-KI	48 ± 10.5 (n = 4)	
β-hydroxybutyrate (mM)	WT	0.122 ± 0.04 (n = 6)	ND
	SIRT1-KI	0.186 ± 0.07 (n = 4)	
Corticosterone (ng mL ⁻¹)	WT	2.63 ± 0.42 (n = 5)	ND
	SIRT1-KI	2.39 ± 0.61 (n = 4)	
IGF-1 (ng mL ⁻¹)	WT	352 ± 11.8 (n = 5)	ND
	SIRT1-KI	324 ± 14.2 (n = 4)	

Total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), adiponectin, leptin, free fatty acids (FFA), triglycerides, and glycerol, IGF-1 were measured in males wild-type (WT) and SIRT1-KI mice at 3, 6 and 12 months of age; and lactate, hydroxybutyrate, and corticosterone were measured in 3-month-old males mice. The number of mice used for each determination is shown in parenthesis.

**P* < 0.05 or less (ANOVA; ND, not determined).

physical function and decreased reproduction (Weindruch & Walford, 1988). One way to assess physical ability is a rotarod test, which is a measure of balance and coordination. CR improves the performance of mice on a rotarod (Weindruch & Walford, 1988), as does the putative SIRT1 activator resveratrol (Baur *et al.*, 2006). We tested SIRT1-KI and littermate controls using an accelerating rotarod assay and found that the SIRT1-KI mice displayed a strikingly improved performance (Fig. 5D).

One possible explanation for the improved physiological and physical functions of SIRT1-KI mice is that they are more active than WT, thereby improving their conditioning. To test this idea, we monitored several parameters of physical activity of WT and SIRT1-KI mice in their home cages. WT and transgenic mice showed no significant difference in six parameters of physical activity, including distance traveled and rearing (Fig. 5E,F and Supplementary Fig. S3). Thus, phenotypes of SIRT1-KI mice cannot be attributed to increased baseline physical activity.

Although SIRT1-KI mice are not sterile, we wished to gauge whether a more subtle effect on mating was caused by the transgene. Thus, we measured the time of first reproduction in WT and SIRT1-KI mice. To maximize the sensitivity of this assay, we first mated the transgene into the C57/B6J background so a comparison could be made in genetically matched animals. Reproduction was significantly delayed in SIRT1-KI transgenic mice compared to WT littermate controls (Fig. 5G).

Discussion

We have generated SIRT1 transgenic mice in which the SIRT1 cDNA has been knocked into the β-actin locus (SIRT1-KI). Interestingly, although this transgene should be ubiquitously expressed, we observed overexpression only in WAT, BAT, brain and MEFs in SIRT1-KI mice compared to WT littermate controls. Overexpression was not observed in muscle and liver, suggesting that the SIRT1-KI allele is not ubiquitously expressed, or SIRT1 protein levels are tightly controlled in certain tissues.

The presence of the SIRT1-KI allele results in several interesting phenotypes, which are robust even in a mixed genetic background. First, transgenic mice are leaner and also have lower levels of adipokines produced by WAT. Second, they display lower levels of blood cholesterol. Third, they show improved glucose homeostasis, as indicated by a reduction in blood insulin and glucose levels. The reduction in insulin is unlikely to be an effect mediated by SIRT1 in β cells, because this sirtuin is a positive regulator of insulin in these cells (Moynihan *et al.*, 2005; Bordone *et al.*, 2006). Moreover, SIRT1-KI mice are more glucose tolerant, further suggesting that their low insulin levels may arise from an increase in insulin sensitivity. Fourth, SIRT1-KI mice are more metabolically active, that is, they eat more and display higher oxygen consumption normalized per body weight.

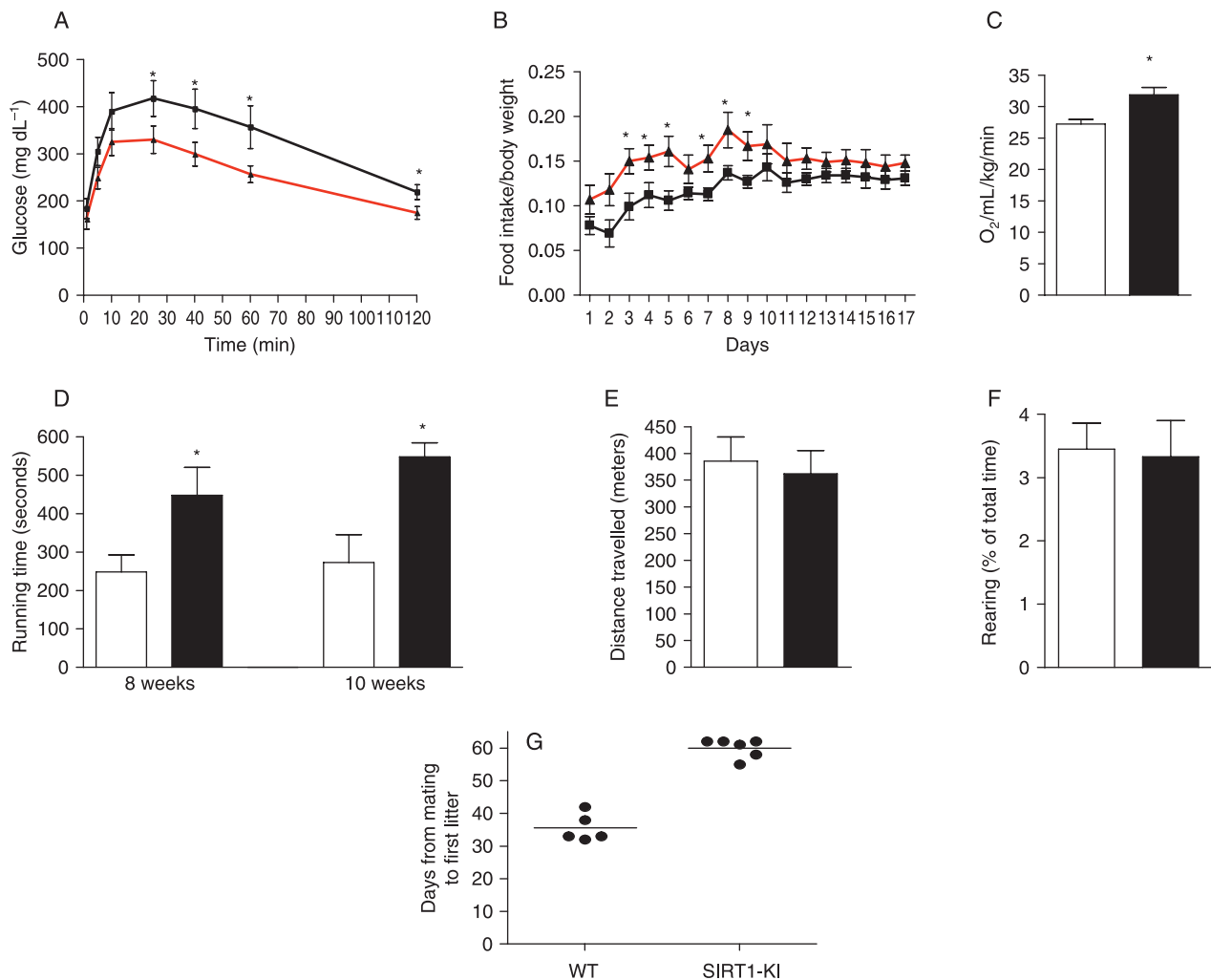


Fig. 5 Metabolic and behavioral analyses of males SIRT1-KI mice. (A) Glucose tolerance test in wild-type (WT) (black line) and SIRT1-KI mice (red line) ($n = 6$). $*P < 0.05$ at 25, 40 60 and 120 min (ANOVA). (B) Food intake is expressed per body weight in WT mice (black line; $n = 6$) and transgenic mice (red line; $n = 6$) as monitored over a 17-day period. (C) Oxygen consumption in WT (white bars) and transgenic mice (black bars) ($*P < 0.02$, ANOVA; $n = 5$). (D) Time to fall from an accelerating rotarod was measured in 8- and 10-week-old mice ($n = 3$ per group; $*P < 0.001$, t -test). Each bar indicates the average of all measurements taken for each genotype group during a given test week, error bar indicates one standard deviation. $*P < 0.001$ by two-tailed homoschedastic Student's t -test. (E) Distance traveled was measured over a 24-h period ($n = 7$ per group). (F) Percentage of rearing time over a 24-h period ($n = 7$ per group). White bar represents WT mice, black bar represents SIRT1-KI mice. (G) C57BL/6J SIRT1-KI animals show a delay in reproductive maturity with respect to WT mice. The difference between groups was found significant, with the $P < 0.0001$ in a one-tailed t -test. SIRT1-KI mice were backcrossed to C57BL/6J four or five generations and littermates were chosen for comparison.

Overexpression of Sir2 extends lifespan in yeast, *C. elegans* and *Drosophila* by a mechanism overlapping moderate CR (Kaeberlein *et al.*, 1999; Tissenbaum & Guarente, 2001; Rogina & Helfand, 2004). In mice, CR not only engenders longevity, but also triggers two important organismal phenotypes, improved physical function and decreased reproduction (Holehan & Merry, 1985a,b). Both of these phenotypes are evident in the SIRT1-KI mice: transgenic mice outperform WT in a rotarod challenge and also show a delay in the age at which they first reproduce. Along with the metabolic changes described above, these findings indicate overlap in the phenotypes of the SIRT1-KI mice and CR.

The fact that some SIRT1-KI metabolic and organismal phenotypes recapitulate some aspects of CR is consistent with the idea that SIRT1 is an important mediator of CR and activation of this sirtuin, which is observed in several tissues of CR animals, is causally associated with at least some of the induced physiological changes.

Experimental procedures

Generation of SIRT1 transgenic mice

The targeting strategy was previously described in Politi *et al.* (2004). The knockin targeting construct was made using mouse

β -actin genomic DNA from *SalI-EcoRI*. An IRES-loxP-neo-loxP cassette was inserted into the *FspI* site located in the 3' untranslated region (UTR) of mouse β -actin gene. Mouse SIRT1 cDNA along with bovine growth hormone polyA was subsequently inserted 3' to the IRES-loxP-neo-loxP cassette. Thus, the expression of Sir2 is driven by β -actin promoter and only occurs after the deletion of neomycin resistance gene by Cre recombinase. The targeting construct was electroporated into embryonic stem cells and neomycin resistant clones were selected and screened by Southern blot using genomic DNA digested by *EcoRI* and *PacI* (WT band, 11.5 kb; mutant band, 8.2 kb). Properly targeted clones were transferred into blastocysts to derive chimeras. Germline transmission was achieved and the heterozygote mice were crossed with Cre-expressing mice to obtain germline deletion of neomycin cassette in the targeted allele. The deletion of neo was confirmed by Southern blot using mouse tail DNA digested with *EcoRI* and *PacI* (WT band, 11.5 kb; mutant band, 6.8 kb). The resulting SIRT1-KI mice in a C57BL/6 and 129/Sv mixed genetic background were bred to generate SIRT1-KI transgenic heterozygotes and their WT littermate controls. Genotypes were routinely determined by polymerase chain reaction (PCR) using primers: actb 5' primer, 5'-tatggaatcctgtggcatcatga-3'; actb 3' primer, 5'-caaagccatgccaatgtgtctct-3'; SIRT1 specific primer, 5'-ggcatgcccagatccaagtta-3'.

Laboratory animals

Mice were housed in groups of three to five in filter-top cages and were given free access to water and normal chow food. The mice were housed under controlled conditions: temperature (25 ± 1 °C) and light cycle (7:00–19:00 hours). Animals were cared for in accordance to the MIT Committee on Animal Care and males were used, unless otherwise noted. All the mice used for the studies were males.

Measurement of protein levels by Western blot

Tissue samples and cells were homogenized on ice in radio-immunoprecipitation assay (RIPA) buffer with a tissue homogenizer (Brinkmann Instruments, Westbury, NY, USA). The RIPA buffer had a final concentration of 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4 , 1 mM NaF, and contained a 1 : 1000 dilution of a protease inhibitor cocktail (CalBiochem, San Diego, CA, USA). The homogenate was centrifuged at 16 000 *g* for 10 min and lysates were transferred to fresh tubes. Protein concentrations were determined by the Bradford method. The lysates were electrophoresed in a 10–15% or 4–12% acrylamide sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel and proteins transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA). The membranes were then blocked in bovine serum albumin (BSA) (Zymed, San Francisco, CA, USA) and probed with polyclonal antibody against SIRT1 (Upstate, New York, NY,

USA), monoclonal antiactin antibody (Chemicon, Temecula, CA, USA), monoclonal β -actin (Abcam, Cambridge, MA, USA), UCP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), sarco actin (Sigma-Aldrich, St. Louis, MO, USA) and tubulin (Abcam) overnight at 4 °C. After washing, membranes were probed with secondary antibody labeled with horseradish peroxidase at room temperature for 1 h. Immunolabeled proteins were then detected by using ECL Plus (GE Healthcare, Piscataway, NJ, USA).

Body weight and food intake

Six-month-old male mice were weighed once a month up to 10 months of age. Food intake and weight were monitored and recorded every day for 17 days.

Body lean mass

Whole body weight of male mice (3 months old) was recorded. Visceral epididymal fat pads and gastrocnemius muscles were carefully removed and weighed.

Oxygen consumption measurement

Animals (males) were acclimated to the respiratory chambers for 1 day before the gas exchange measurement. Mice were individually housed in the home cages, which were put in the metabolic chamber (Kent Scientific, Litchfield, CT, USA). Data on oxygen (O_2) exchange was collected for 24 h. The O_2 sensor is directly connected to an amplifier for recording. The chamber is equipped with a water bottle and a food tray. Air reference values were measured before every measurement. The data are expressed as percentage of oxygen consumed * vol of chamber/kg/min.

Glucose tolerance test

Six-month-old fasted male mice (14 h) were given an intraperitoneal glucose load (1 g glucose kg^{-1} body weight using a solution of 10% glucose in physiological saline). Blood was collected at 0, 5, 10, 25, 40, 60 and 120 min and used for glucose measurement (Onetouch, Milpitas, CA, USA). Insulin (Ultra-sensitive Mouse Insulin EIA; ALPCO Diagnostic, Windham, NH, USA) measurements were done the night before the food was removed and the day before the glucose injection. Access to food was denied during the course of the study.

In vivo insulin measurement

Six-month-old males mice were subjected to overnight fast followed by intraperitoneal glucose injection (1 g kg^{-1} body weight). Blood samples were collected from the tail vein the night before the experiment (fed), and right before the injection (fasted). Plasma glucose was measured using OneTouch according to manufacturer's specifications. Insulin levels were measured using the Ultrasensitive Mouse Insulin EIA according to manufacturer's specifications (ALPCO Diagnostic).

Hormone determination

Fluorescent assays were used to measure total cholesterol, total triglycerides, glycerol and free fatty acid concentrations in the mouse plasma (Amano Enzymes, Inc., Elgin, IL, USA). HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], ATP (adenosine 5'-triphosphate), hydrogen peroxide and MgCl₂ were purchased from Sigma-Aldrich, while Amplex Red was purchased from Molecular Probes (Invitrogen). Adiponectin (detecting all forms except the monomer) was measured using the single plex kit from LINCO Research (Billerica, MA, USA). Lactate, LDL and HDL cholesterol were measured according to manufacturer's directions following National Committee for Clinical Laboratory Standards guidelines using an AU400e@ Chemistry Immuno Analyzer (Olympus America Inc., Center Valley, PA, USA). Leptin, IGF-1 and corticosterone were measured using an enzyme immunoassay from LINCO accordingly to manufacturer's specifications. For all experiments, blood was collected in the morning from male mice at the indicated ages.

Rotarod analysis

Rotarod training and testing were performed based on established protocols (Hockly *et al.*, 2003). After acclimatization to the rotarod apparatus (Columbus Instruments, Columbus, OH, USA) at the age of 6 weeks, three mice per genotype group were tested twice per test day each week. The rotarod accelerated from 4 to 40 r.p.m. over the course of each 600-s test. Mice were allowed to rest 10 min between trials.

Behavioral analysis

Home cage behavioral analysis was performed essentially as described in (Steele *et al.*, 2007). Briefly, 8-week-old male mice were singly housed for at least 1 week prior to being video recorded in a normal home cage for 24 h (a complete light-dark cycle). Analysis of the videos was performed by HomeCageScan software 2.0 (Clever Sys, Reston, VA, USA) and data are represented as averages \pm standard error of the mean across SIRT1-KI or WT control mice ($n = 7$ per group).

Mating analysis

Six-week-old SIRT1-KI or WT animals (two males and four females) were mated to 8- to 10-week-old C57BL/6J partners and the number of days between mating to the birth of first litter was recorded.

Statistical analysis

Results are shown as the mean \pm SEM. Statistical analysis was performed by ANOVA using GraphPad Prism. The threshold for statistical significance was set at $P < 0.05$.

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References

- Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R, Sinclair DA (2006) Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **444**, 337–342.
- Bordone L, Guarente L (2005) Calorie restriction, SIRT1 and metabolism: understanding longevity. *Nat. Rev. Mol. Cell Biol.* **6**, 298–305
- Bordone L, Motta MC, Picard F, Robinson A, Jhala US, Apfeld J, McDonagh T, Lemieux M, McBurney M, Szilvasi A, Easlson EJ, Lin S-J, Guarente L (2006) Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic β cells. *PLoS Biol.* **4**, e31.
- Chen D, Steele AD, Lindquist S, Guarente L (2005) Increase in activity during calorie restriction requires Sirt1. *Science* **310**, 1641.
- Cheng HL, Mostoslavsky R, Saito S, Manis JP, Gu Y, Patel P, Bronson R, Appella E, Alt FW, Chua KF (2003) Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. *Proc. Natl Acad. Sci. USA* **100**, 10794–10799.
- Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. (2004) Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* **305**, 390–392.
- Despres JP, Lemieux I (2006) Abdominal obesity and metabolic syndrome. *Nature* **444**, 881–887.
- Hockly E, Woodman B, Mahal A, Lewis CM, Bates G (2003) Standardization and statistical approaches to therapeutic trials in the R6/2 mouse. *Brain Res. Bull.* **61**, 469–479.
- Holehan AM, Merry BJ (1985a) Modification of the oestrous cycle hormonal profile by dietary restriction. *Mech Ageing Dev* **32**, 63–76.
- Holehan AM, Merry BJ (1985b) The control of puberty in the dietary restricted female rat. *Mech Ageing Dev* **32**, 179–191.
- Kaeberlein M, McVey M, Guarente L (1999) The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev* **13**, 2570–2580.
- Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P, Auwerx J (2006) Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1 α . *Cell* **127**, 1109–1122.
- Lamming DW, Wood JG, Sinclair DA (2004) Small molecules that regulate lifespan: evidence for xenohormesis. *Mol. Microbiol.* **53**, 1003–1009.
- Lin SJ, Ford E, Haigis M, Liszt G, Guarente L (2004) Calorie restriction extends yeast life span by lowering the level of NADH. *Genes Dev.* **18**, 12–16.
- Lin SJ, Kaeberlein M, Andalis AA, Sturtz LA, Defossez PA, Culotta VC, Fink GR, Guarente L (2002) Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature* **418**, 344–348.
- McBurney MW, Yang X, Jardine K, Hixon M, Boekelheide K, Webb JR,

- Lansdorp PM, Lemieux M (2003) The mammalian SIR2 α protein has a role in embryogenesis and gametogenesis. *Mol. Cell. Biol.* **23**, 38–54.
- Moynihan KA, Grimm AA, Plueger M, Bernal-Mizrachi E, Ford E, Cras-Méneur C, Permutt M, Imai S (2005) Increased dosage of mammalian Sir2 in pancreatic β cells enhances glucose-stimulated insulin secretion in mice. *Cell Metab.* **2**, 105–117.
- Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO (2005) Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science* **310**, 314–317.
- Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, de Oliveira RM, Leid M, McBurney MW, Guarente L (2004) Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR- γ . *Nature* **429**, 771–776.
- Politi K, Kljuic A, Szabolcs M, Fisher P, Ludwig T, Efstratiadis A (2004) 'Designer' tumors in mice. *Oncogene* **23**, 1558–1565.
- Qiang L, Wang H, Farmer SR (2007) Adiponectin secretion is regulated by SIRT1 and the endoplasmic reticulum oxidoreductase Ero1- α . *Mol. Cell. Biol.* **27**, 4698–4707.
- Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P (2005) Nutrient control of glucose homeostasis through a complex of PGC-1 α and SIRT1. *Nature* **434**, 113–118.
- Rogina B, Helfand SL (2004) Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc. Natl Acad. Sci. USA* **101**, 15998–16003.
- Steele AD, Jackson WS, King OD, Lindquist S (2007) The power of automated high-resolution behavior analysis revealed by its application to mouse models of Huntington's and prion diseases. *Proc. Natl Acad. Sci. USA* **104**, 1983–1988.
- Tissenbaum HA, Guarente L (2001) Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature* **410**, 227–230.
- Weindruch R, Walford RL (1988) *The Retardation of Aging and Disease by Dietary Restriction*. Springfield, IL: Charles C Thomas.
- Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D (2004) Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* **430**, 686–689.

Supplementary material

The following supplementary material is available for this article:

Fig. S1 Genotypes of progeny of SIRT1-KI/+ \times +/+ mice are tabulated. Significant deviation from the expected 1 : 1 ratio was observed, as indicated.

Fig. S2 (A) Western blot of SIRT1 in liver and skeletal muscle in wild-type (WT) and SIRT1-KI littermates. Each lane represents an individual mouse. (B) Western blot for β -actin, total actin and tubulin in liver, skeletal muscle and white adipose tissue (WAT) of wild-type (WT) and transgenic littermates. Sarco-actin was also blotted in muscle.

Fig. S3 Hanging, jumping and walking was measured in 8- and 10-week-old mice over a 24-h period ($n = 7$ per group). White bar represents wild-type (WT) mice, black bar represents SIRT1-KI mice.

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