Supporting Information

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SI Materials and Methods

Materials. Recombinant porcine growth hormone (GH) was obtained from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Disorders, National Institutes of Health. Resveratrol, β-NAD, nicotinamide, and trichostatin A were purchased from Sigma-Aldrich, and sirtinol was purchased from Calbiochem.

Cell Culture, Primary Hepatocyte Isolation, and Plasmid and siRNA Transfection. HepG2 cells and HEK293 cells were purchased from American Type Culture Collection and cultured in growth medium containing DMEM without phenol red, 10% FBS, 1.0 g/L D(+)-glucose, 100 U/mL penicillin G, and 100 mg/mL kanamycin at 37 °C in a 5% CO_2 atmosphere. Primary hepatocytes were prepared by nonrecirculating collagenase perfusion through the portal vein of rats anesthetized with pentobarbital (60 mg/kg), as described previously (1). Cells were seeded at a density of 1.8×10^6/mL on collagen-coated dishes in serum-free DMEM/Ham's F-12 medium supplemented with penicillin/streptomycin (100 U/mL and 100 mg/mL, respectively), hydrocortisone (5×10^{-8} M), insulin (1.75×10^{-7} M), L-ornithine (4×10^{-4} M), L-lactic acid (1.77×10^{-5} M), selenium (2.5×10^{-8} M), and ethanolamine (1×10^{-6} M). Cultures were maintained at 37 °C in a humidified incubator in an atmosphere containing 5% CO_2.

After 48 h of starvation, cells were stimulated with porcine GH for 15 min at different concentrations, and the lysates were subjected to immunoblotting. pcDNA3.1 FLAG-tagged mouse signal transducer and activator of transcription (STAT) 5B (a kind gift from D. Wang, The University of Alabama, Tuscaloosa, AL), pcDNA1 human GH receptor (GHR) (2), pcDNA3.1 FLAG-tagged human SIRT1 WT (Addgene), pcDNA3.1 FLAG-tagged human SIRT1 H363Y (Addgene), pGEX3 GST-tagged and pGEX5X-2 GST-tagged human SIRT1 (a kind gift from Dr. F. Ishikawa, Kyoto University, Kyoto, Japan) were used for the experiments. STAT5B lysine (Lys) mutants K681A, K694A, K701A, and K705A were produced from pcDNA3.1 FLAG-tagged mouse STAT5B using a Stratagene site-directed mutagenesis kit. The STAT5 deletion mutants were constructed using PCR and the pcDNA 3.1 Directional TOPO Expression Kit (Life Technologies). Transfection of the plasmid was performed in HepG2 and HEK293 cells using Lipofectamine 2000 (Life Technologies) and in primary rat hepatocytes using the JetPEI hepatocyte (for overexpression) and INTERFERin (for knockdown) transfection reagents (Polypus Transfection). Human and rat SIRT1 siRNA were used as described previously (3, 4). The siRNA sequences were as follows: human SIRT1 siRNA, 5′-GAAGTTGACCTCCTCATTGT-3′; rat SIRT1 siRNA, 5′-AAGTGCCTCAAATATTAATAA-3′. For knockdown of SIRT1, HepG2 cells were transfected with siRNA using Lipofectamine RNAi MAX (Life Technologies). Rat primary hepatocytes were transfected with siRNA using INTERFERin (Polyplus Transfection).

Animals. Mouse experiments were performed according to the guidelines of the Animal Ethics Committee of Kobe University Graduate School of Medicine. Male C57BL/6J mice (age 6–8 wk) and male Sprague–Dawley rats (age 8 wk) were purchased from CLEA Japan. Hypophysectomized male C57BL/6J Jms Slc mice (age 8 wk) were purchased from Japan SLC. The mice were kept on a 12-h day/night cycle and had free access to water and normal chow unless indicated otherwise.

Antisense Oligonucleotide Knockdown. Male C57BL/6J Jms Slc mice were divided into control and SIRT1 antisense oligonucleotide (ASO) groups. SIRT1 ASO or control ASO (Vivo-Morpholino; Gene Tools) was administered daily for 3 d (10 mg/kg body weight, i.v.) (5). The hypophysectomized mice were treated as well. The experimental design is shown in Fig. S1.

Antibodies. Antibodies recognizing acetylated Lys (9441, 1:1,000; Cell Signaling Technology), phospho-STAT5 (Tyr694; 9359, 1:1,000; Cell Signaling Technology), STAT5 (C-17; sc-835, 1:1,000; Santa Cruz Biotechnology), SIRT1 (sc-15404, 1:500; Santa Cruz Biotechnology), SIRT1 (07-131, 1:1,000; Millipore), Janus kinase 2 (JAK2; 06-1310, 1:1,000; Millipore), phosphotyrosine (clone PY20; 05-947, 1:500; Millipore), GHR (ab78426, 1:500; Abcam), phospho-MAPK (422705, 1:500; Calbiochem), ERK1 (sc-94, 1:500; Santa Cruz Biotechnology), V5 antibody (R960-25, 1:1,000; Life Technologies), and FLAG (M2-HRP conjugate; A8592, 1:1,000; Sigma-Aldrich) were used for immunoblot analysis.

Immunoprecipitation and Immunoblot Analysis. Immunoprecipitation was performed as described previously (6), with minor modifications. HEK293 cells, rat primary hepatocytes, and mice livers were lysed in buffer containing 50 mM Tris (pH 7.6), 250 mM NaCl, 10 mM 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 10 mM nicotinamide, 1 μM trichostatin A (Sigma-Aldrich), a protease inhibitor mixture (Roche), and a phosphatase inhibitor mixture (Nacalai Tesque). The lysates were sonicated and centrifuged at $15{,}000 \times g$ for 20 min. Once protein concentrations were normalized, the supernatant was incubated with the indicated antibody overnight at 4 °C. The samples were then incubated with Protein G Sepharose 4 Fast Flow (GE Healthcare). After an additional washing, the precipitates were heat-denatured in sample buffer. For immunoblot analysis, proteins were separated by SDS/PAGE and transferred to a PVDF membrane. The membranes were blocked with Blocking One or Blocking One-P (Nacalai Tesque), incubated overnight with primary antibody at 4 °C, washed, incubated with the secondary antibody for 1 h at room temperature, and then washed again. The signals were visualized using a Chemi-Lumi One L (Nacalai Tesque) or Immunostar LD (Wako) substrate. Signals were densitometrically quantified with the ImageQuant LAS 4000 imaging system (GE Healthcare).

Measurement of Serum Insulin-Like Growth Factor I Levels by ELISA. The Quantikine ELISA Kit (R&D Systems) was used to measure total insulin-like growth factor 1 (IGF-I) concentrations in the mice. All sample measurements were performed in duplicate.

Measurement of NAD /NADH Ratio. NAD and NADH levels were determined according to instructions provided with the Abcam NAD/NADH Assay Kit.

GST Pull-Down Experiments. SIRT1 GST fusion protein was expressed in *Escherichia coli* and purified using the GST Bulk Kit (GE Healthcare) according to the manufacturer's instructions. Recombinant GST fusion proteins were incubated with in vitro-translated FLAG-tagged STAT5B protein. The protein complexes were then pulled down with GST beads, eluted with SDS sample buffer, and resolved by SDS/PAGE. The GST proteins were detected by Coomassie brilliant blue staining.

Luciferase Reporter Gene Assay. HEK293 cells were transiently transfected with pcDNA3.1 GHR and lactogenic hormone-

responsive element (pLHRE) containing the luciferase reporter plasmid (a kind gift from Dr. T. A. Willson, Walter and Eliza Hall Institute of Medical Research, Bundoora, Australia) and pcDNA3.1 STAT5B WT, or STAT5B Lys mutant (K681, K694, K701, or K705). After 12 h, the cells were stimulated with 100 ng/mL GH and then incubated for an additional 36 h. Whole-cell extracts were collected and subjected to a luciferase assay using the Promega Luciferase Assay System, according to the manufacturer's instructions.

Quantitative Real-Time PCR. Total RNA was extracted from cells using TRIzol reagent (Life Technologies), and cDNA was synthesized using the ReverTra Ace qPCR RT Kit (Toyobo). Real-time PCR was performed using SYBR Premix Ex Taq (Perfect Real Time; TaKaRa) and detected using an Applied Biosystems 7500 Real-Time PCR System, according to the manufacturer's instructions. The following primer sequences were used for real-time PCR: human IGF-I: forward, 5′-GGAAGTACATTTGAAGAACGCAAGT-3′, reverse, 5′-ATCCTGCGGTGGCATGTC-3′; rat IGF-I: forward, 5′-CATGCCCAAGACTCAGAAGG-3′, reverse, 5′-CGTGGCATTTTCTGTTCCTC-3′; mouse IGF-I: forward, 5′-GCTTGCTCACCTTCACCAGC-3′, reverse, 5′-AATGTACTTCCTTCTGAGTCT-3′; β-actin: forward, 5′-TCACCCTGAAGTACCCCATC-3′, reverse, 5′-GGGGTGTTGAAGGTCTCAAA-3′; human SIRT1: forward, 5′-AAATGCTGGCCTAATAGAGTGG-3′, reverse, 5′-TGGTGGCAAAAACAGATACTGA-3′; rat GHR: forward, 5′-CCCGGTTCTGCAAAGAATTA-3′, reverse, 5′-AACGACACTTGGTGAATCGAG-3′; mouse IGFBP-3: forward, 5′-GACGACGTACATTGCCTCAG-3′, reverse, 5′-GTCTTTTGTGCAAAATAAGGCATA-3′; mouse ALS: forward, 5′-GGCCAGCTCTGTACAAGGAA-3′, reverse, 5′-CAGAAAGCCAGAAGCACCAC-3′; mouse SOCS2: forward, 5′-CGCGAGCTCAGTCAAACA-3′, reverse, 5′-GAATGCGAACTATCTCTAATCAAGAA-3′.

Statistical Analysis. All data are reported as mean ± SEM. Statistical analyses were performed using the Student *t* test for comparison of two groups and one-way ANOVA with Scheffé's *F* test for multiple group comparisons. A P value < 0.05 was considered statistically significant. Representative results from at least three independent experiments are presented unless stated otherwise.

1. Thissen JP, Pucilowska JB, Underwood LE (1994) Differential regulation of insulin-like growth factor I (IGF-I) and IGF-binding protein-1 messenger ribonucleic acids by amino acid availability and growth hormone in rat hepatocyte primary culture. *Endocrinology* 134(3):1570–1576.
2. Iida K, et al. (1999) Functional characterization of truncated growth hormone (GH) receptor-(1-277) causing partial GH insensitivity syndrome with high GH-binding protein. *J Clin Endocrinol Metab* 84(3):1011–1016.
3. Cohen HY, et al. (2004) Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 305(5682):390–392.
4. Li Y, Xu W, McBurney MW, Longo VD (2008) SirT1 inhibition reduces IGF-I/IRS-2/Ras/ERK1/2 signaling and protects neurons. *Cell Metab* 8(1):38–48.
5. Nie Y, et al. (2009) STAT3 inhibition of gluconeogenesis is downregulated by SirT1. *Nat Cell Biol* 11(4):492–500.
6. Handayaningsih AE, et al. (2011) Reactive oxygen species play an essential role in IGF-I signaling and IGF-I-induced myocyte hypertrophy in C2C12 myocytes. *Endocrinology* 152(3):912–921.

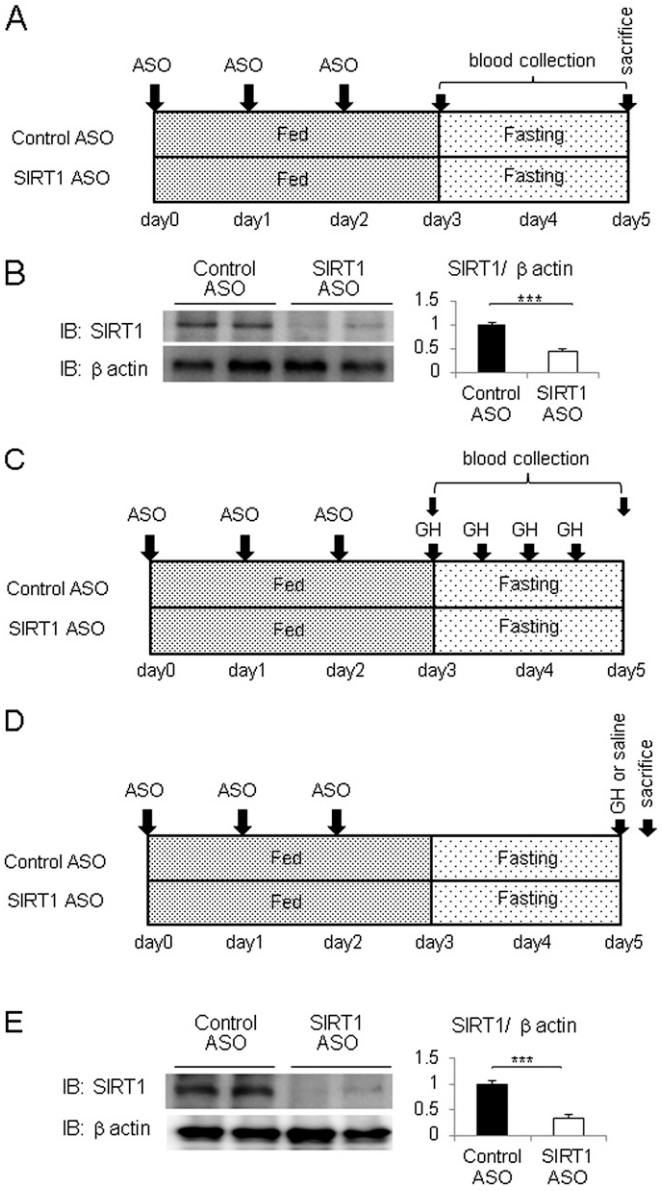

Fig. S1. Experimental design for SIRT1 knockdown and GH administration in vivo. (*A*) Experimental design for SIRT1 knockdown in vivo. (*B*) SIRT1 protein levels were significantly reduced in the liver. (*C* and *D*) Experimental designs for the analysis of serum IGF-I concentrations (*C*) and GH signals (*D*) in hypophysectomized mice. (*E*) SIRT1 protein levels were significantly reduced in the livers of hypophysectomized mice.

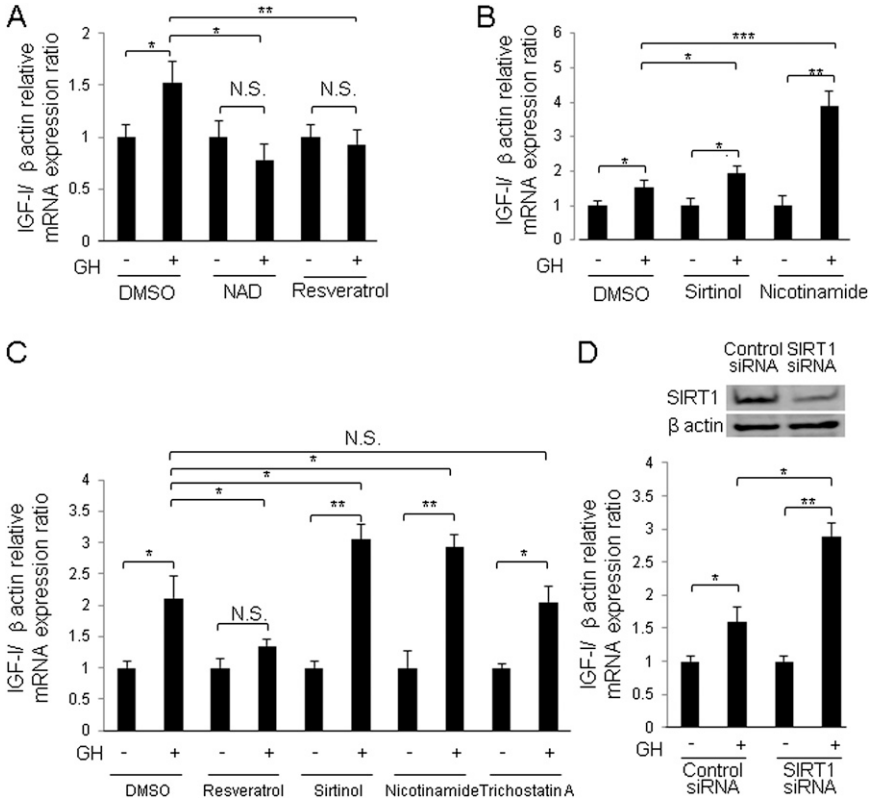

Fig. S2. Involvement of SIRT1 in GH-induced IGF-I mRNA production. The effect of the changes in SIRT1 activity on GH-dependent IGF-I mRNA production was evaluated by quantitative real-time PCR in human hepatocellular carcinoma cell line HepG2 and primary rat hepatocytes. HepG2 cells (*A*, *B*, and *D*) were stimulated with GH (500 ng/mL) for 6 h, and rat primary hepatocytes (*C*) were stimulated with GH (500 ng/mL) for 24 h. (*A*) Pretreatment of SIRT1 activators (10 mM NAD for 6 h, 100 μM resveratrol for 6 h). (*B*) Pretreatment of SIRT1 inhibitors (50 mM sirtinol for 6 h, 10 mM nicotinamide for 6 h). (*C*) Pretreatment of SIRT1 activator (100 μM resveratrol for 24 h) or inhibitors (50 μM sirtinol for 24 h, 10 mM nicotinamide for 24 h), and class I and II histone deacetylase inhibitor (1 μM trichostatin A for 24 h). (*D*) Knockdown of SIRT1 by siRNA in HepG2 cells. IGF-1 mRNA expression levels were quantified by quantitative real-time PCR and normalized to those of β-actin. The results are mean ± SEM of six samples per group, expressed relative to the control group. $*P < 0.05$; $**P < 0.01$. N.S., not significant.

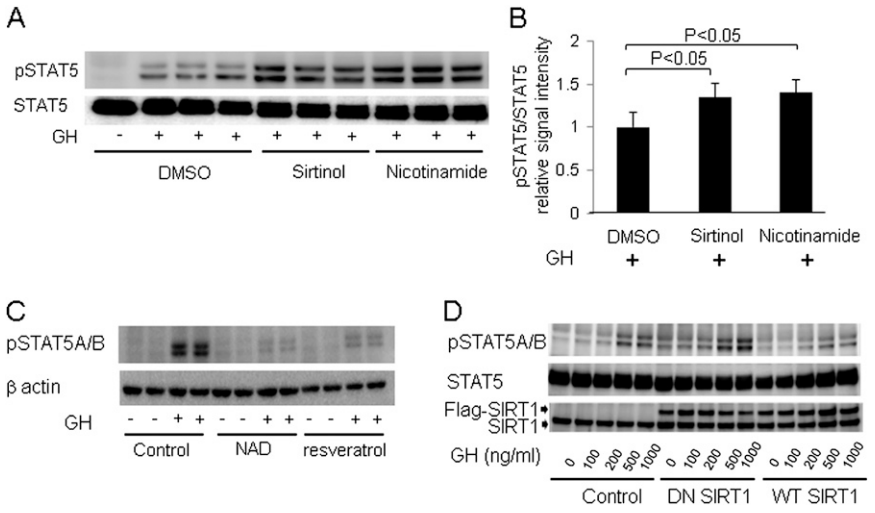

Fig. S3. GH-induced tyrosine (Tyr) phosphorylation of STAT5 in after treatment with SIRT1 inhibitors and activators. GH-stimulated Tyr phosphorylation of STAT5 was analyzed by immunoblotting. Primary rat hepatocytes were stimulated with GH for 15 min, after which the lysate was subjected to immunoblotting. (*A* and *B*) Pretreatment of SIRT1 inhibitor (50 μM sirtinol or 10 mM nicotinamide for 6 h). (*C*) Pretreatment of SIRT1 activator (10 mM NAD for 1 h, 100 μM resveratrol for 6 h). (*D*) Overexpression of WT SIRT1 or DN SIRT1.

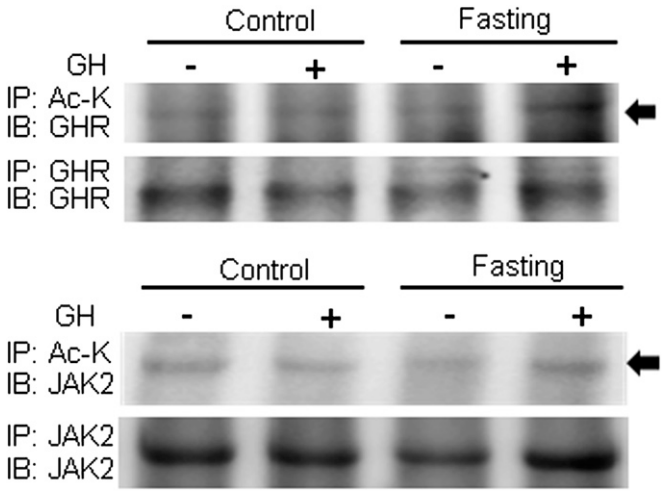

Fig. S4. Fasting did not affect the Lys acetylation status of GHR and JAK2 in the liver. Male C57BL/6J mice were fasted for 24 h. GH (3 mg/kg, i.p.) was administered, and the mice were killed 15 min later, after which liver protein lysates were subjected to immunoblot analysis.

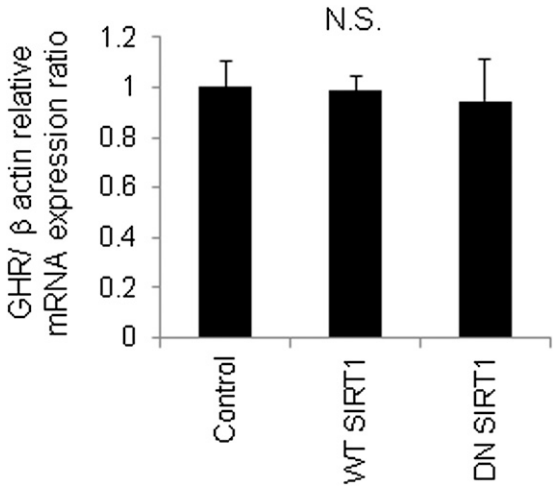

Fig. S5. GHR expression is not affected by SIRT1 activity. GHR mRNA levels in primary rat hepatocytes were measured by quantitative real-time PCR and normalized to β-actin levels. N.S., not significant.