Supporting Information

Lee et al. 10.1073/pnas.1310331110

SI Materials and Methods

Animals and Diets. All mice were housed in a mouse facility with a 12-h light/dark cycle in a temperature-controlled room. Creation of adipocyte protein 2 cre (aP2-cre) (1) and short stature homeobox gene 2 (*Shox2*) floxed (2) mice has previously been described. Control ($Shox2^{f/f}$) and fat-specific *Shox2* knockout (F-$Shox2^{-/-}$) mice were maintained on a standard chow diet containing 22% calories from fat, 23% from protein, and 55% from carbohydrates (Mouse Diet 9F 5020; PharmaServ), or subjected to high fat diet (HFD) containing 60% calories from fat, 20% from protein, and 20% from carbohydrates (OpenSource Diet D12492; Research Diet) beginning at ∼6 wk of age. Mice were allowed ad libitum access to water and food. Animal care and study protocols were approved by the Animal Care Committee of Joslin Diabetes Center and were in accordance with the National Institutes of Health guidelines.

Metabolic Analysis. For metabolic analysis, 8-wk-old mice (F-$Shox2^{-/-}$ and controls), which had been on HFD for 2 wk, were housed individually and evaluated for ambulatory activity using an OPTO-M3 sensor system [Comprehensive Laboratory Animal Monitoring System (CLAMS); Columbus Instruments], which counts beam breaks for 60 s for each mouse, five times per hour during two full light/dark cycles. Indirect calorimetry was measured on the same mice using an open-circuit Oxymax system (Columbus Instruments). After a 48-h acclimation period, exhaust air was sampled for 60 s every 12 min in each cage consecutively for 72 h in the fed state for the determination of O_2 and CO_2.

Fed glucose was measured between 9:00 AM and 11:00 AM in tail vein blood samples (Ascensia Elite). Insulin was measured using rat insulin ELISA with mouse standards (Crystal Chem). Leptin and adiponectin were also assessed by ELISA (Crystal Chem). Intraperitoneal glucose (2 g/kg weight) and insulin tolerance (1.25 units/kg) tests were performed in unrestrained conscious mice after a 16- and 4-h fast, respectively. Glucose and insulin tolerance tests were performed on mice at 14 wk of age after 8 wk of HFD exposure.

Measurement of Adipocyte Size. Perigonadal and s.c. fat from mice on 12 wk of HFD (started at 6 wk) ($n = 4$ per group) was fixed in 10% (vol/vol) formalin and paraffin embedded; 8-μm sections were hematoxylin/eosin stained. Five digital images (20×) from nonoverlapping fields were taken from each slide (total 20 fields per group), and adipocyte diameters were calculated using Image J software.

Lipogenesis and Lipolysis in Isolated Adipocytes. Subcutaneous and perigonadal adipose tissue from mice were quickly dissected, minced, and digested with 1 mg/mL collagenase I (Worthington Biosciences) in DMEM containing high glucose (4,500 mg/L) supplemented with 1% fatty acid free BSA shaking for 45 min at 37 °C. Cells were passed through a 200-μM filter and washed twice with Krebs Ringer Hepes buffer containing 1% BSA. Lipogenesis and lipolysis assays were performed as previously described (3, 4).

Gene Expression. Analysis of gene expression was conducted using quantitative PCR (qPCR). Total RNA was extracted using an RNeasy minikit (QIAGEN), and 3 μg was reverse transcribed in 100 μl using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Five microliters of diluted cDNA from the reverse transcription reaction (1/10) was amplified with specific primers (300 nM each) in a 10-μL PCR with an SYBR green PCR master mix (Applied Biosystems). Analysis of gene expression was done in an ABI Prism 7900 sequence detector with an initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles, each cycle consisting of 95 °C for 15 s and 60 °C for 1 min. SYBR green fluorescence emission was monitored after each cycle. For each gene, mRNA expression was calculated relative to Tata binding protein (Tbp). Amplification of specific transcripts was confirmed by melting-curve profiles at the end of each PCR.

Human Adipose Tissue Collection. Paired samples of visceral and s.c. adipose tissue were obtained from 198 Caucasian men ($n = 99$) and women ($n = 99$) who underwent open abdominal surgery for gastric banding, cholecystectomy, appendectomy, weight reduction surgery, abdominal injury, or explorative laparotomy. Of these patients, 21 females and 15 males had type 2 diabetes mellitus (T2DM). The T2DM patients were excluded from further analysis. All subjects had stable body weight with no fluctuations of >2% of the body weight for at least 3 mo before surgery. The age of the subjects ranged from 24 to 85 y for males and from 27 to 86 y for females. Body mass index (BMI) ranged from 21.7 to 46.8 kg/m^2 for the males and from 20.8 to 54.1 kg/m^2 for the females. Waist-hip ratio (WHR) ranged from 0.8 to 1.37 for the males and from 0.62 to 1.45 for the females. Visceral fat area and the relative ratio of intraabdominal visceral fat to s.c. fat area were determined using computed tomography (CT) scans at the level of L4–L5 with an attenuation range of −30 to −190 Houndsfield units as previously described (5). Obese (BMI > 30) with a preferential s.c. distribution was defined as a ratio of s.c. fat area to visceral fat area of >0.4 whereas a preferential visceral distribution was defined as a ratio of s.c. fat area to visceral fat area of <0.4. Samples of visceral and s.c. adipose tissue were immediately frozen in liquid nitrogen after removal. The study was approved by the ethics committee of the University of Leipzig. All subjects gave written informed consent before taking part in the study.

Western Blot. Proteins were extracted from cells in radioimmunoprecipitation assay buffer (RIPA) buffer with 0.1% SDS. Thirty micrograms of protein was subjected to SDS/PAGE and transferred to polyvinylidine fluoride membranes, and blots were probed with the (1:1,000) anti-beta3 adrenergic receptor (Adrb3) (Novus Biologicals), anti-phosphorylated hormone senstive lipase (pHSL), anti-hormone senstive lipase, or anti-perilipin (Abcam).

Retroviral and Lentiviral Infection. *Shox2* was stably overexpressed in C3H10T1/2 cells by retroviral infection. Phoenix cells were grown to 70% confluency in 10-cm plates and were transiently transfected with 1 μg of retroviral expression vectors pBABE-Empty-bleo or pBABE-*Shox2*-bleo using Superfect (Qiagen) according to the manufacturer's instructions. At 48 h after transfection, virus-containing medium was collected and passed through a 0.45-μm pore size syringe filter. Filter-sterilized Polybrene (hexadimethrine bromide; 8 μg/mL) was added to the virus-loaded medium, and the medium was then applied to proliferating (20% confluent) cells. At 48 h after infection, cells were treated with trypsin and replated in a medium supplemented with zeocin (Invitrogen) as a selection antibiotic.

shRNA knockdown of Shox2 was achieved in C3H10T1/2 cells by lentiviral infection. Plates (10 cm) of 70% confluent 293FT cells were transiently transfected with 1 μg of lentiviral pSUPER green fluorescent protein (shGFP) or pSUPER shShox2 and the viral packaging vectors SV-E-MLV-env and SV-E-MLV using Superfect (Qiagen) according to the manufacturer's instructions.

Forty-eight hours after transfection, virus-containing medium was collected and passed through a 0.45-μm syringe filter. Filter-sterilized Polybrene (hexadimethrine bromide, 8 μg/mL) was added to the virus-loaded medium, and the medium was applied to proliferating cells. Forty-eight hours after infection, cells were treated with trypsin and replated in a medium supplemented with puromycin (Invitrogen) as a selection antibiotic.

Cells were grown in DMEM containing high glucose (4,500 mg/L) with 10% FBS (Gemini). Differentiation was induced by induction media:growth media supplemented with 1 μM dexamethasone, 500 μM isobutylmethylxanthine, and 100 nM insulin, for 2 d. Except for the assessment of differentiation capacity, the peroxisome proliferator-activated receptor gamma (PPARγ) agonist rosiglitazone (1 μM) was added in all experiments. Cells were then grown for 6 d in growth media with 100 nM insulin. Medium was replaced every other day.

Oil Red O Staining. Liver samples from four F-Shox2$^{-/-}$ and control mice after 12 wk of HFD were embedded in optimal cutting temperature (OCT) compound and sectioned 9–10 μM thick. Differentiated culture plates were washed in PBS, and cells were fixed in 4% paraformaldehyde for 1 h. Oil Red O of liver samples and plates was performed by diluting a stock solution (0.5 g of Oil Red O [Sigma]) in 100 mL of isopropanol with water [60:40 (vol/vol)], followed by filtration. After staining, samples were washed several times in water. Liver sections were lightly counterstained with hematoxylin. Pictures are taken at 20 ×.

Reporter Assays. NIH 3T3 and C3H10T1/2 were plated in 24-well plates and grown to 70% confluence. The cells were transfected with 1 μg of a previously described 5.13-kb *Adrb3* luciferase reporter (6), pcDNA3.1-Empty, or pcDNA3.1-CCAAT/enhancer binding protein alpha (C/EBPα), pBABE-Shox, or BABE-Empty, and pRenilla-TK (Promega), using Superfect transfection reagent (Qiagen). The cells were harvested 24 h later, and luciferase activity was measured using a Dual Renilla Luciferase II Assay Kit, and normalized to Renilla luciferase measurements (Promega).

Immunoprecipitation. C3H10T1/2 were plated in 10-cm plates and grown to 80% confluence. The cells were transfected with a total of 10 μg of plasmid DNA composed of different combinations of pcDNA3.1-C/EBPα, pBABE-Shox2-FLAG, or BABE-Empty using Superfect transfection reagent (Qiagen). Cells were lysed in immunoprecipitation (IP) Lysis Buffer (Pierce) supplemented with protease and phosphatase inhibitors (Sigma) and passed through a 29-gauge syringe five times. One milligram of protein was used for each immunoprecipitation with 2 ug of anti-C/EBPalpha (Santa Cruz; sc61), anti-FLAG (Sigma; M2), or anti-phosphoserine (Cell Signaling) antibodies. Micro MACS Protein A and Protein G (Miltenyi Biotec) were used for the immunoprecipitation according to the manufacturer's instructions.

Chromatin Immunoprecipitation. C3H10T1/2 cells were grown to 70% confluence in 10-cm plates and transiently transfected with 1 μg of Empty Vector, Shox2-FLAG, and/or C/EBPα for 48 h. Cross-linking was performed by adding 37% formaldehyde to a final concentration of 1% formaldehyde and incubation at room temperature for 10 min and stopped by addition of 10× 1.25 M glycine. Cells were then scraped, and samples were prepared with EZ ChIP (Upstate) according to the manufacturer's protocol. DNA was sonicated on ice using 5× 10-s bursts of 30% output of a Tekmar Sonicator. The chromatin fractions were incubated in each case with 10 μg of one of the following antibodies: anti-C/EBPα (Santa Cruz), and normal rabbit IgG at 4 °C overnight. Immunoprecipitated DNA was analyzed by qPCR detecting the mouse Adrb3 promoter (forward, 5′- GGAGCAATGACTTCTAAACGTACAA-3′; reverse, 5′-TGACCCTTCTAGATGAACACACA -3′).

Statistics. All differences were analyzed by ANOVA or Student *t* test. Results were considered significant if $P < 0.05$.

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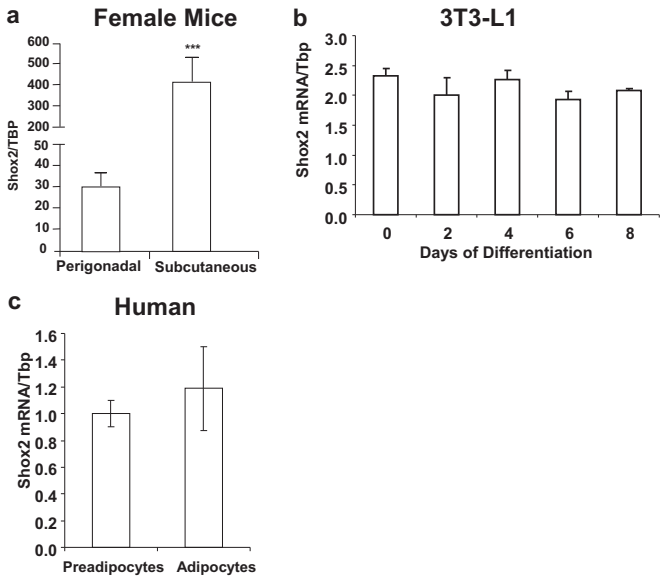

Fig. S1. *Shox2* expression during 3T3-L1 differentiation and in sorted cells. (*A*) Expression level of *Shox2* mRNA was compared using quantitative real-time PCR (qPCR) of RNA isolated from fat pads of 6-wk-old female C57BL/6 animals. Data are normalized to the expression of the housekeeping gene Tata binding protein (Tbp) and shown as mean ± SEM of five samples. (*B*) qPCR analysis of *Shox2* expression in 3T3-L1 cells during in vitro differentiation. Data are shown as mean ± SEM of four replicates. The entire experiment was repeated twice. (*C*) Expression level of SHOX2 mRNA was compared using qPCR of RNA isolated from primary human subcutaneous preadipocytes before and after differentiation into adipocytes.

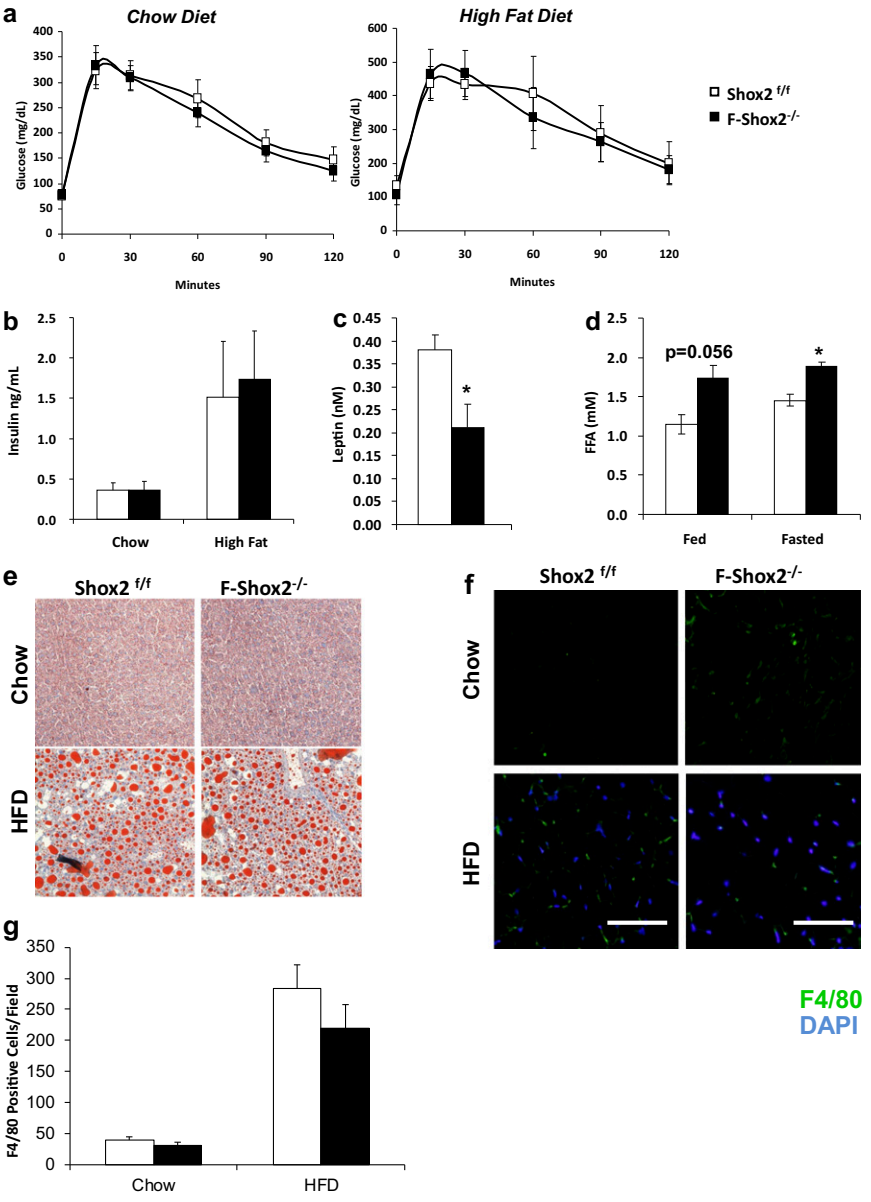

Fig. S2. Effects of fat-specific *Shox2* ablation on glucose tolerance and circulating adipokine and fatty acid levels. (*A*) Glucose tolerance testing of male F-Shox2$^{-/-}$ and control mice on chow diet and 8 wk after HFD exposure. Data are shown as mean ± SEM (n = 5–7 animals per group). (*B*) Circulating insulin levels of male F-Shox2$^{-/-}$ and control mice on chow diet and after 8 wk of HFD exposure were measured by ELISA. Data are shown as mean ± SEM (n = 5–7 animals per group). (*C*) Circulating leptin levels of fasted male and female F-Shox2$^{-/-}$ and control mice after 8 wk of HFD exposure were measured by ELISA. Data are shown as mean ± SEM (n = 5–7 animals per group). *$P < 0.05$ for all panels. (*D*) Circulating free fatty acid (FFA) levels of fed and fasted male F-Shox2$^{-/-}$ and control mice after 8 wk of HFD exposure were measured by ELISA. Data are shown as mean ± SEM (n = 5–7 animals per group). (*E*) Oil Red O staining of male F-Shox2$^{-/-}$ and control livers on chow diet and 12 wk after HFD exposure. Nuclei are counterstained with hematoxylin. Representative digital images (20×) are shown. (*F*) Immunofluorescence for the macrophage marker F4/80 on perigonadal fat of F-Shox2$^{-/-}$ and control mice at 6 wk of age and after 12 wk of HFD exposure. Representative digital images (20×) are shown. (*G*) Quantification of immunofluorescence for the macrophage marker F/480 on perigonadal fat of F-Shox2$^{-/-}$ and control mice at 6 wk of age and after 12 wk of HFD exposure. Four digital images (20×) from nonoverlapping fields were taken from each slide (total 12 fields per group).

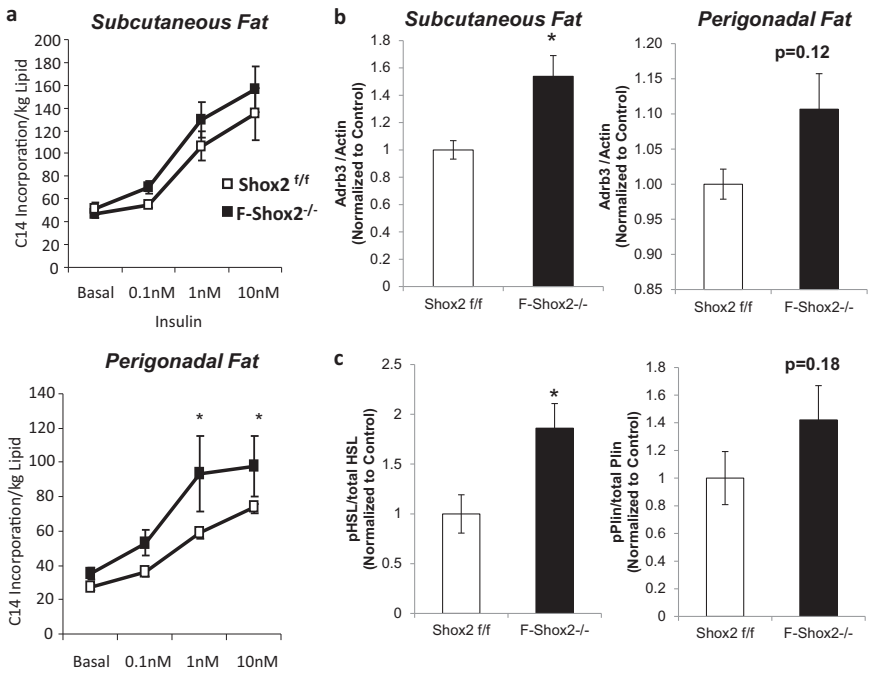

Fig. S3. Effects of fat-specific *Shox2* ablation on lipogenesis. (*A*) Lipogenesis rate as measured by incorporation of [^{14}C] D-glucose into fatty acid from isolated s.c. and perigonadal adipocytes. Adipocytes were isolated from 8-wk-old mice fed HFD for 2 wk. Data are mean ± SEM (n = 4 animals per group). (*B*) Quantitation of Western blots shown in Fig. 3*C* of the β3-adrenergic receptor from protein extracts from the s.c. and perigonadal fat in 8-wk-old male Shox2$^{f/f}$ and F-Shox2$^{-/-}$ mice. Western blot of actin was used as a loading control. Data are mean ± SEM (n = 3 animals per group). (*C*) Quantitation of Western blots shown in Fig. 3*D* of the phosphorylated and total hormone sensitive lipase, and perilipin, and immunoprecipitation with an anti-phospho-serine antibody followed by immunoblotting for perilipin from protein extracts from the s.c. and perigonadal fat in 8-wk-old male Shox2$^{f/f}$ and F-Shox2$^{-/-}$ mice. Western blot of actin was used as a loading control. Data are mean ± SEM (n = 3 animals per group).

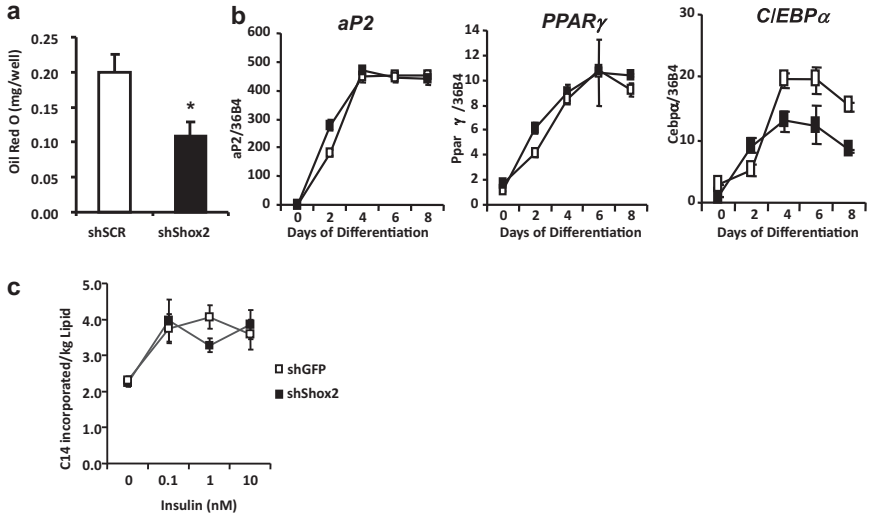

Fig. S4. Lipogenesis and adipogenesis are not changed in sh*Shox2* adipocytes. (*A*) Quantification of Oil Red O staining in shShox2 stably transfected and control C3H10T1/2 cells after adipocyte differentiation. Data shown as mean ± SEM of triplicate samples and repeated twice. (*B*) mRNA was isolated from sh*Shox2* and shGFP stably transfected C3H10T1/2 cells during adipocyte differentiation. Expression of *PPARγ*, *aP2*, and *C/EBPα*, was measured by qPCR at day 0, 2, 4, 6, and 8. Data shown as mean ± SEM of triplicate samples and repeated three times. (*C*) Lipogenesis in isolated sh*Shox2* adipocytes. Lipogenesis rates as measured by incorporation of [^{14}C] D-glucose into fatty acid from shShox2 and control adipocytes after 8 d of differentiation. Data are shown as mean ± SEM of three replicates. The entire experiment was repeated twice. shSCR, shScramble.

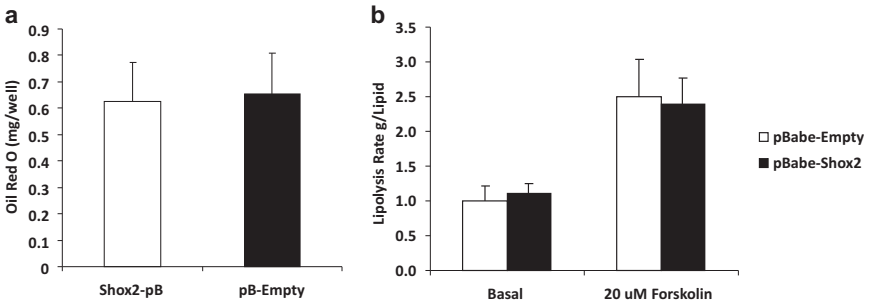

Fig. S5. Lipid accumulation and maximal lipolytic rate are not affected by overexpression of *Shox2*. (*A*) Quantification of Oil Red O staining of stably transfected pBABE-Shox2 and control C3H10T1/2 cells after adipocyte differentiation. Data shown as mean ± SEM of triplicate samples and repeated twice. (*B*) Lipolysis of pBABE-Shox2 stably transfected and control C3H10T1/2 cells after treatment with 20 μM forskolin. Data shown as mean ± SEM of triplicate samples and repeated twice.