Shox2 is a molecular determinant of depot-specific adipocyte function

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Visceral and s.c. fat exhibit different intrinsic properties, including rates of lipolysis, and are associated with differential risk for the development of type 2 diabetes. These effects are in part related to cell autonomous differences in gene expression. In the present study, we show that expression of *Shox2* (Short stature homeobox 2) is higher in s.c. than visceral fat in both rodents and humans and that levels are further increased in humans with visceral obesity. Fat-specific disruption of *Shox2* in male mice results in protection from high fat diet-induced obesity, with a preferential loss of s.c. fat. The reduced adipocyte size is secondary to a twofold increase in the expression of β3 adrenergic receptor (*Adrb3*) at both the mRNA and protein level and a parallel increase in lipolytic rate. These effects are mimicked by knockdown of *Shox2* in C3H10T1/2 cells. Conversely, overexpression of *Shox2* leads to a repression of *Adrb3* expression and decrease lipolytic rate. Shox2 does not affect differentiation but directly interacts with CCAAT/enhancer binding protein alpha and attenuates its transcriptional activity of the Adrb3 promoter. Thus, *Shox2* can regulate the expression of *Adrb3* and control the rate of lipolysis and, in this way, exerts control of the phenotypic differences between visceral and s.c. adipocytes.

developmental genes | adipose depots | lipid metabolism

Obesity, especially central obesity in which the excessive weight is associated with increased amounts of intraabdominal or visceral fat, is associated with insulin resistance, type 2 diabetes and increased risk of cardiovascular disease (1, 2). On the other hand, peripheral obesity, characterized by increased amounts of s.c. fat, is associated with improved insulin sensitivity and lowered risk of type 2 diabetes and atherosclerosis, and appears to even reduce the risk of diseases associated with central obesity (2, 3). Consistent with these findings, we and others have demonstrated beneficial effects of s.c. fat through transplantation studies (4, 5).

These clear differences between s.c. and visceral fat suggest that, in addition to their anatomical location, these fat depots have intrinsic differences at the cellular level. These studies have demonstrated major functional and gene expression differences between s.c. and visceral fat (6, 7). Both isolated adipocytes and the stromovascular fraction containing preadipocytes show cell autonomous differences in the expression of developmental patterning genes between s.c. and visceral depots, suggesting different developmental origins for these fat depots (8).

One of the developmental genes that show differential expression between adipose depots in both mice and humans is Short stature homeobox 2 (*Shox2*). *Shox2* mRNA levels are 20-fold higher in the s.c. vs. the visceral adipose depots. This increased level of *Shox2* expression is independent of leptin levels or diet-induced obesity and is found in both isolated adipocytes, as well as in preadipocytes sorted from the stromovascular fraction, of s.c. vs. visceral fat (9, 10). *Shox2*, formerly known as aOg12 (mice), Prx3 (rats), and SHOT (human), is a homolog to the short stature homeobox gene *SHOX* in humans. *Shox2* is the only *Shox* gene present in mice because mice lack a direct ortholog to human *SHOX*. In mice, ablation of *Shox2* causes embryonic lethality at midgestation due to cardiac and vascular defects (11). Studies of *Shox2*-null and conditional knockout mice have shown an indispensable role of *Shox2* in the formation of the proximal portion of the limb skeleton and synovial joints (12, 13). The shortened limbs observed in Turner syndrome may be related to loss of *SHOX* (14, 15).

Due to the striking differences in expression of *Shox2* between the s.c. and visceral fat depots, as well as its known roles in regulating the development of mesodermally derived tissues, we hypothesized that *Shox2* may have a potential role in the differential biology and function in adipocytes from these different depots. In the current study, we find that *SHOX2* expression is higher in the s.c. than visceral adipose tissue in mice and humans. *SHOX2* expression in the s.c. fat of people with visceral obesity is increased compared with lean individuals and individuals with s.c. obesity. After ablation of *Shox2* in adipocytes of mice (F-Shox2$^{-/-}$), the resultant male mice are resistant to diet-induced obesity and exhibit decreased adipose mass, especially in the s.c. depot. Adipocytes isolated from male F-Shox2$^{-/-}$ mice have an increase in catecholamine-induced lipolysis with a corresponding increase in β3-adrenergic receptor (*Adrb3*) expression. Knockdown and overexpression studies in C3H10T1/2 cell lines demonstrate that *Shox2* is able to repress transcriptional activation of CCAAT/enhancer binding protein alpha (C/EBPα) on the *Adrb3* promoter in a dose-dependent manner. Thus, *Shox2* acts to control differential adipocyte function and plays an important role in the depot-specific differences in adipocyte behavior.

Results

Shox2 Expression Is Correlated with Visceral Obesity in Humans. We have previously shown that *Shox2* is differentially expressed in s.c. and intraabdominal fat of mice and humans (8). *Shox2* levels are highest in the s.c. fat depots, with only low levels of expression in brown fat or visceral fat depots (9). Likewise, preadipocytes sorted from the stromovascular fraction of s.c. fat have *Shox2* levels that are 95-fold higher than those found in visceral fat (10). Quantitative real-time PCR (qPCR) analysis of *Shox2* mRNA from s.c. and perigonadal adipose tissue of obesity-prone C57BL/6 mice confirmed an ∼20-fold higher level of *Shox2* mRNA in s.c. versus perigonadal fat in both male and female mice (Fig. 1*A*; Fig. S1*A*). Similar differential expression of *Shox2* was observed in the adipose depots of obesity-resistant 129/SvEv mice, with no significant differences between these two mouse strains in *Shox2* expression (Fig. 1*A*).

A similar, but even more striking, differential expression was observed in adipose tissue of humans. Thus, qPCR analysis of *SHOX2* gene expression in 163 nondiabetic Caucasian subjects

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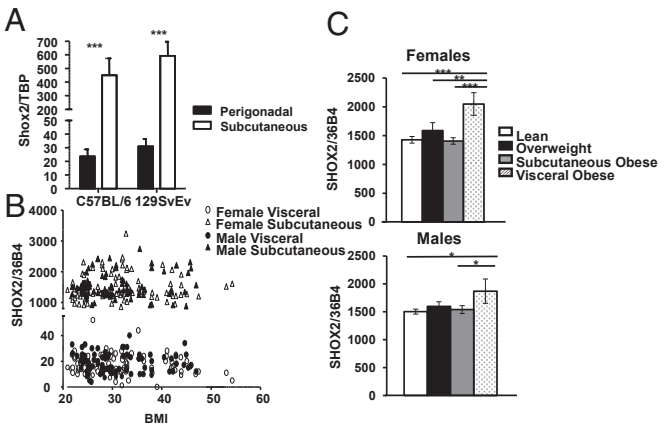

Fig. 1. The expression of *Shox2* in different mouse and human fat depots. (*A*) Expression level of *Shox2* mRNA was compared using quantitative real-time PCR (qPCR) of RNA isolated from fat pads of 6-wk-old male C57BL/6 and 129SvEv animals. Data are normalized to Tata binding protein (Tbp) and shown as mean ± SEM of five samples. (*B*) qPCR analysis of *SHOX2* expression from s.c. and visceral fat of female and male human subjects plotted versus BMI. Data are normalized to the expression of the housekeeping gene acidic ribosomal phosphoprotein P0 (36B4). (*C*) qPCR analysis of *SHOX2* expression from s.c. fat of male and female lean, overweight, s.c. obese and viscerally obese patients. Subject characteristics and visceral and s.c. obesity defined as described in *SI Materials and Methods*. Data are normalized to the expression of 36B4 and shown as mean ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

revealed on average 83-fold higher levels of *SHOX2* mRNA in s.c. fat than visceral fat (18.9 ± 0.6 arbitrary units vs. 15,521.7 ± 31.4, $P < 10^{-97}$). This differential expression was observed over the entire range of BMI from 20 to >50 kg/m^2 and in both males and females (Fig. 1*B*). Although there was no correlation between *SHOX2* expression with blood glucose levels, age, or sex, there was a significant correlation between *SHOX2* expression and the site of fat deposition in obese individuals. Thus, when the study group was divided into four subgroups—lean [body mass index (BMI) < 25], overweight (BMI 25–30), obese (BMI > 30) with a preferential s.c. distribution, and obese with a preferential visceral distribution—*SHOX2* expression in s.c. fat was significantly higher in both obese women and men with a preferential visceral distribution compared with lean, overweight, and obese subjects with an s.c. distribution (Fig. 1*C*). *Shox2* mRNA levels were not changed during differentiation of either mouse or human preadipocytes (Fig. S1 *B* and *C*). Thus, in both rodents and in humans, *Shox2* is expressed in adipocyte and their precursor cells and is differentially expressed between fat depots, and, in humans, *SHOX2* expression in the s.c. fat is higher in men and women with visceral obesity than those with s.c. obesity or those with no obesity.

Ablation of Shox2 Attenuates Diet-Induced Obesity. To investigate the role of *Shox2* in adipocyte biology, we created mice with an ablation of *Shox2* in adipocytes using the aP2-cre transgenic mouse and mice carrying a *Shox2* allele with locus of X-over P1 (loxP) sites surrounding the entire coding sequence. Adipocyte protein 2 cre (aP2-cre); Shox2$^{f/f}$ (termed F-Shox2$^{-/-}$) mice exhibited efficient and specific ablation of Shox2 in fat, were born at the expected Mendelian ratio, and exhibited no overt abnormalities (16).

F-Shox2$^{-/-}$ and littermate control Shox2$^{f/f}$ animals were fed a standard chow (21% fat by calories) diet or high fat (60% fat) diet starting at age 6 wk. On a chow diet, there was no difference in body weight in F-Shox2$^{-/-}$ males followed out to 11 mo of age. By contrast, on high fat diet (HFD), F-Shox2$^{-/-}$ males gained weight at a significantly slower rate than controls, with a 29% weight reduction in weight gain over the 12 wk (Fig. 2*A*). This reduction corresponded to a 53% and 30% decrease of the s.c. and perigonadal fat depots in the F-Shox2$^{-/-}$ mice as early as 6 wk after exposure to HFD (Fig. 2*B*). As a result, the relative ratio of perigonadal to s.c. flank fat after 12 wk of HFD was 2.45 in male F-Shox2$^{-/-}$ mice verses a ratio of only 1.33 in control mice (Fig. 2*C*). There were no observable changes in triglyceride accumulation in the brown fat of F-Shox2$^{-/-}$ mice after HFD. Likewise, there was no difference in adipocyte size in the perigonadal fat between knockout and control mice (Fig. 2*D*). However, adipocyte size in the s.c. fat of F-Shox2$^{-/-}$ mice was markedly reduced, with a 37% decrease in median adipocyte diameter, indicating that the decrease in fat mass was due to a decrease in cell size, rather than a decrease in cell number (Fig. 2*E*). Thus, F-Shox2$^{-/-}$ mice are partially protected against diet-induced obesity due to a decrease in adipocyte hypertrophy, particularly in the s.c. depot.

F-Shox2$^{-/-}$ Mice Are Not Protected Against Diet-Induced Insulin Resistance. HFD induces insulin resistance in mice. This insulin resistance is reflected by an increase in blood glucose levels and levels of circulating insulin. As expected, control Shox2$^{f/f}$ mice showed a 57% increase in fasting and 42% increase in peak glucose levels during the glucose tolerance test after 8 wk of HFD compared with chow diet-fed mice. Despite the resistance of male F-Shox2$^{-/-}$ mice to HFD-induced obesity, these mice showed a similar 40% increase in fasting blood glucose and a 41% increase in peak glucose levels during a glucose tolerance test (Fig. S2*A*) compared with chow diet-fed mice. Circulating insulin levels were similarly increased by HFD in male control (0.36 ng/mL to 1.51 ng/mL) and F-Shox2$^{-/-}$ (0.36 ng/mL to 1.73 ng/mL) mice (Fig. S2*B*).

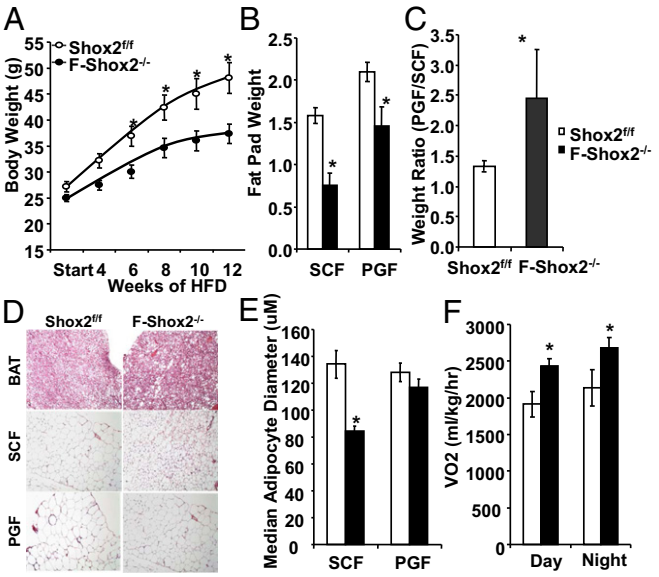

Fig. 2. F-Shox2$^{-/-}$ males are resistant to HFD-induced obesity. (*A*) Body weight measurement of F-Shox2$^{-/-}$ and control animals during 12 wk on HFD (started at 6 wk of age). Data are shown as mean ± SEM of five to seven animals per group. $*P < 0.05$ for all panels. (*B*) Weight of fat pads from F-Shox2$^{-/-}$ and control males after 6 wk of HFD exposure. Data are shown as mean ± SEM (n = 4–5). (*C*) Ratio of perigonadal (intraabdominal) fat/flank (s.c.) fat pad weights for F-Shox2$^{-/-}$ and control males after 6 wk of HFD exposure. Data are shown as mean ± SEM (n = 4–5). (*D*) Hematoxylin/eosin-stained representative sections from F-Shox2$^{-/-}$ and control males after 12 wk of HFD exposure. Pictures were taken at 200× . (*E*) Calculation of median adipocyte size in perigonadal and s.c. fat from F-Shox2$^{-/-}$ and control males after 12 wk of HFD exposure. Values represent median ± SEM of five digital images from four animals per group. (*F*) Oxygen consumption rate in the light and dark cycles of male F-Shox2$^{-/-}$ and control animals after 2 wk of HFD feeding. Data are shown as mean ± SEM (n = 4). BAT, brown adipose tissue; PGF, perigonadal fat; SCF, subcutaneous fat; VO_2, oxygen uptake.

Reflecting the changes in fat mass, F-Shox2$^{-/-}$ mice had a 48% reduction in circulating leptin levels compared with controls after 12 wk of HFD (Fig. S2*C*). Thus, even though F-Shox2$^{-/-}$ mice are protected against diet-induced obesity, the reduced body weight did not lead to protection against diet-induced glucose intolerance or hyperinsulinemia.

Although F-Shox2$^{-/-}$ and control male mice consumed similar amounts of calories (3.9 ± 0.5 g per mouse per day and 4.3 ± 0.5 g per mouse per day, respectively) and had comparable levels of activity, indirect calorimetry of male mice maintained on the HFD for 2 wk revealed that F-Shox2$^{-/-}$ mice had an ∼20% increase in oxygen consumption during both the light period (2,431 ± 102 in F-Shox2$^{-/-}$ versus 1,913 ± 171 mL·kg^{-1}·h^{-1}, $P < 0.05$) and dark period (2,687± 133 versus 2,136 ± 240 mL·kg lean^{-1}·h^{-1}, $P < 0.05$) (Fig. 2*F*). This change in oxygen consumption occurred with no change in the respiratory quotient, an indicator of the relative rates of fat and carbohydrate oxidation. Together, these data suggest that the protection from diet-induced obesity exhibited by F-Shox2$^{-/-}$ mice was due to increased energy expenditure.

***Shox2* Ablation Leads to an Increase in Lipolytic Rate.** Decreased adipocyte size, i.e., reduced triglyceride content, can result from a defect in adipocyte differentiation, decreased lipogenesis, or increased lipolysis. qPCR analysis of mRNA isolated from s.c. fat, perigonadal fat, and brown adipose tissue from F-Shox2$^{-/-}$ and littermate controls revealed no significant differences in the levels of known regulators of adipogenesis, indicating that *Shox2* ablation does not impair adipocyte differentiation. However, after exposure to HFD for 2 wk, adipocytes isolated from the s.c. and perigonadal depots of F-Shox2$^{-/-}$ mice revealed an increase in catecholamine-induced lipolytic rate. Thus, when stimulated with isoproterenol at concentrations of 0.1 and 1μM, F-Shox2$^{-/-}$ adipocytes, particularly those from s.c. fat, exhibited a >50% increase in glycerol release compared with control cells (Fig. 3*A*). This increase in lipolytic rate led to a 51% and 30% increase in circulating free fatty acids (FFA) in F-Shox2$^{-/-}$ on HFD in the fed and fasted states compared with controls (Fig. S2*D*). Despite the increase in circulating FFA levels, ectopic fat deposition in the liver was not visibly increased (Fig. S2*E*), presumably due to protection from the increased energy expenditure. Although increased lipolysis has been shown to increase recruitment of adipose tissue macrophages (17), immunofluorescence on hisotological sections for the macrophage marker F4/80 and quantification of macrophage number showed that macrophage density was not significantly increased in the s.c. fat of F-Shox2$^{-/-}$ males on chow or HFD (Fig. S2 *F* and *G*). In adipocytes from the perigonadal fat, but not the s.c. fat, of F-Shox2$^{-/-}$ mice, there was also increased insulin-stimulated lipogenesis (60% at 1 nM and 32% at 10 nM insulin) compensating for the increased lipolysis and helping to maintain adipocyte size (Fig. S3*A*).

Numerous proteins, especially adipose triglyceride lipase (*Atgl*) and hormone sensitive lipase (*Hsl*), work coordinately to break down and metabolize stored triglycerides. The expression of the two rate-limiting enzymes of lipolysis, *Atgl* and *Hsl*, or the key colipase comparative gene identification-58 (CGI-58) were not significantly changed in adipocytes of F-Shox2$^{-/-}$ mice on HFD (Fig. 3*B*). However, expression of the β3-adrenergic receptors (*Adrb3*) was increased by ∼50% in F-Shox2$^{-/-}$ adipocytes isolated from the s.c. fat of mice on HFD (Fig. 3*B*). Levels of mRNA for the *Adrb3* were also increased in F-Shox2$^{-/-}$ perigonadal adipocytes, but this difference did not reach statistical significance. These differences were paralleled by differences in Adrb3 protein levels in s.c. and perigonadal fat of F-Shox2$^{-/-}$ mice on HFD (Fig. 3*C*, Fig. S3*B*). Catecholamines stimulate lipolysis mainly through the phosphorylation and subsequent activation of hormone sensitive lipase (Hsl) and perilipin (Plin1). Western blot of extracts from s.c. fat of F-Shox2$^{-/-}$ mice on HFD showed a significant

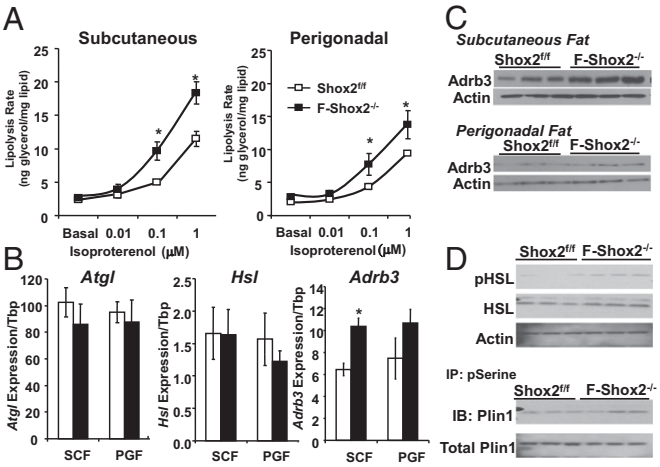

Fig. 3. *Shox2* ablation leads to an increase in lipolytic rate on HFD. (*A*) Lipolysis as measured by glycerol release from isolated s.c. and perigonadal adipocytes of F-Shox2$^{-/-}$ males and controls after 2 wk of HFD exposure. Data are shown as mean ± SEM of four animals per group normalized by lipid content of the cells. The entire experiment was repeated twice. *$P <$ 0.05 for all panels. (*B*) Expression levels of *Hsl*, *Atgl*, and *Adrb3* mRNA were compared using qPCR of mRNA from perigonadal and s.c. fat in 8-wk-old male Shox2$^{f/f}$ and F-Shox2$^{-/-}$ mice after 2 wk of HFD. Data are shown as mean ± SEM ($n = 4$). (*C*) Western blot 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. Mice were subjected to HFD for 2 wk. Western blot of actin was used as a loading control. (*D*) Western Blots of the phosphorylated (pHSL) and total hormone sensitive lipase, and perilipin, and immunoprecipitation (IP) with an anti-phospho-serine (pSerine) antibody followed by immunoblotting (IB) for perilipin from protein extracts from the s.c. and perigonadal fat in 8-wk-old male Shox2$^{f/f}$ and F-Shox2$^{-/-}$ mice. Mice were subjected to HFD for 2 wk.

1.9-fold increase in phosphorylation of Hsl and a trend for increased Plin1 phosphorylation (1.4-fold; $P = 0.18$) with no changes in total protein or mRNA levels (Figs. 3*D* and S3*C*). Thus, ablation of *Shox2* leads to increased levels of *Adrb3*, which results in increased catecholamine-induced lipolysis, especially in s.c. fat cells, leading to smaller adipocyte size in F-Shox2$^{-/-}$ mice on HFD.

shRNA Knockdown of *Shox2* Increases *Adrb3* Expression and Lipolysis. To further investigate the mechanisms underlying the increased lipolysis found in F-Shox2$^{-/-}$ adipocytes, we used an shRNA lentivirus to stably knockdown endogenous *Shox2* expression in adipocytes differentiated from C3H10T1/2 mesenchymal stem cells (sh*Shox2* cells). qPCR analysis of sh*Shox2* cells showed that *Shox2* message was decreased by at least 80% both in the undifferentiated state and after differentiation into adipocytes using the standard induction protocol with insulin, dexamethasone, and isobutylmethylxanthine. sh*Shox2* cells displayed a marked reduction of lipid accumulation upon Oil Red O staining (Fig. S4*A*). qPCR analysis of the major regulators and markers of differentiation, including *C/EBPα*, peroxisome proliferator-activated receptor gamma (*PPARγ*), and *aP2*, showed no differences between sh*Shox2* cells and control cells over 8 d of differentiation (Fig. S4*B*). Thus, the decreased lipid accumulation in *Shox2*-deficient cells is due to changes in lipid metabolism and not a defect in differentiation.

In control and sh*Shox2* cells, basal and insulin-induced lipogenic rates measured using [^{14}C] D-glucose incorporation into fatty acids showed no significant differences (Fig. S4*C*). Lipolysis rate, as measured by the rate of glycerol release, was also not different in the basal condition. However, upon stimulation of lipolysis with 10 nM to 100 nM isoproterenol, sh*Shox2* cells exhibited a

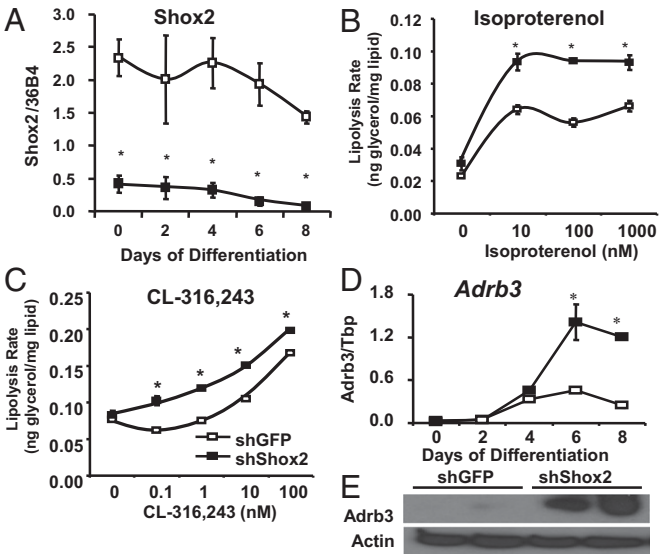

Fig. 4. shRNA knockdown of *Shox2* increases *Adrb3* expression and lipolysis. (*A*) Expression level of *Shox2* mRNA was compared by qPCR between C3H10T1/2 cells stably transfected with sh*Shox2* and shGFP (control) during adipocyte differentiation. Data shown as mean ± SEM of triplicate samples and repeated three times. **P* < 0.05 for all panels. (*B*) Lipolysis rates after stimulation with isoproterenol as measured by glycerol release from sh*Shox2* and shGFP C3H10T1/2 adipocytes after 8 d of differentiation. Data are shown as mean ± SEM of three replicates and normalized by lipid content of the cells. The entire experiment was repeated twice. (*C*) Lipolysis rates after stimulation with CL 316,243 as measured by glycerol release from sh*Shox2* and shGFP C3H10T1/2 adipocytes after 8 d of differentiation. Data are shown as mean ± SEM of three replicates and normalized by lipid content of the cells. The entire experiment was repeated twice. (*D*) mRNA was isolated from sh*Shox2* and shGFP stably transfected C3H10T1/2 cells during adipocyte differentiation. *Adrb3* expression was measured by qPCR. Data shown as mean ± SEM of triplicate samples and repeated three times. (*E*) Western blot of the β3-adrenergic receptor from protein extracts from sh*Shox2* and shGFP C3H10T1/2 adipocytes after 6 d of differentiation. Western blot of actin was used as a loading control.

40%–68% increase compared with controls (Fig. 4*B*). Additionally, when lipolysis was stimulated by CL-316,243, a specific activator of *Adrb3*, *Shox2* knockdown C3H10T1/2 cells demonstrated an increased lipolytic rate across a broad concentration range from 0.1 to 100 μM (Fig. 4*C*). In agreement with the in vivo phenotype observed in the F-Shox2$^{-/-}$ mice, qPCR analysis demonstrated that knockdown of *Shox2* in C3H10T1/2 led to a 2.5-fold increase in *Adrb3* mRNA (Fig. 4*D*), and this difference was confirmed by Western blotting, which showed a dramatic up-regulation of Adrb3 protein in knockdown cells (Fig. 4*E*). Thus, *Shox2* does not affect differentiation or lipogenesis but leads to an increase in *Adrb3* expression that, in turn, causes a significant increase in catecholamine-induced lipolysis.

Overexpression of *Shox2* Decreases Lipolysis and *Adrb3* Expression. To further elucidate the role of *Shox2* in adipocytes, we created C3H10T1/2 cells with stable overexpression of *Shox2* (Fig. 5*A*). These cells differentiated into adipocytes normally with normal lipid accumulation (Fig. S5*A*) and expression of differentiation markers. Mirroring the findings of increased *Adrb3* in the F-Shox2$^{-/-}$ mice and the knockdown cell line, qPCR analysis C3H10T1/2 adipocytes overexpressing *Shox2* exhibited a 58% decrease of *Adrb3* mRNA expression (Fig. 5*B*) and a parallel decrease in *Adrb3* protein (Fig. 5*C*). This decrease in *Adrb3* expression lead to an ∼50% decrease in isoproterenol-stimulated lipolytic rate at all concentrations tested, with no change in basal lipolytic rate (Fig. 5*D*). Likewise, although maximal lipolytic rate after 20 μM forskolin stimulation was unchanged (Fig. S5*B*), lipolysis stimulated by the β3-specific agonist, CL-316,243 was decreased by about 50% in *Shox2* overexpressing C3H10T1/2 adipocytes across a wide range of concentrations from 0.1 to 1,000 nM (Fig. 5*E*). Taken together with the data from the fat-specific *Shox2* knockout mice and the *Shox2* knockdown cell line, these data indicate that *Shox2* is a negative regulator of *Adrb3* expression and lipolytic rate both in vivo and in vitro.

Shox2 Represses C/EBPα Transactivation of *Adrb3*-luc Constructs. The β3-adrenergic receptor has been shown to play an important role in adipocyte function and metabolic homeostasis (18). The fact that ablation of *Shox2* causes increased expression of *Adrb3* both in vivo and in vitro, and that overexpression led to decreased *Adrb3* expression, prompted us to test whether *Shox2* might directly regulate *Adrb3* expression. A reporter construct harboring a 5.13-kb upstream fragment of the mouse *Adrb3* gene linked to a luciferase reporter gene (*Adrb3*-luc) (19) was cotransfected with different concentrations of Shox2 into the C3H10T1/2 cells. Luciferase assays demonstrated that basal *Adrb3* promoter activity in C3H10T1/2 cells was very low under these conditions, and no change in activity was seen following Shox2 expression (Fig. 6*A*). However, the *Adrb3* promoter is potently activated by C/EBPα (20), and, when C/EBPα was cotransfected with the reporter construct, there was a dose-dependent increase in luciferase levels by 2-, 11- and 19-fold at 10 ng, 50 ng, and 200 ng of C/EBPα,

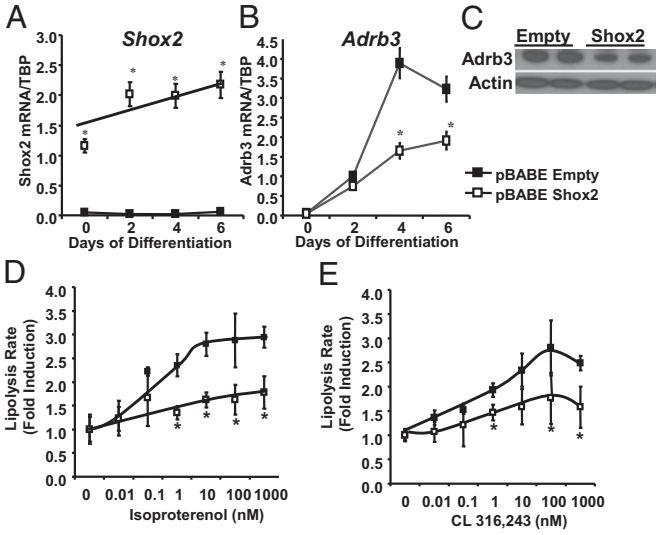

Fig. 5. Overexpression of *Shox2* decreases lipolysis and *Adrb3* expression. (*A*) Expression level of *Shox2* mRNA was compared by qPCR between C3H10T1/2 cells stably transfected with pBABE-*Shox2* and pBABE-Empty (control) during adipocyte differentiation. Data are mean ± SEM of triplicate samples and the experiment was repeated three times. **P* < 0.05 for all panels. (*B*) Expression level of *Adrb3* mRNA was compared by qPCR between C3H10T1/2 cells stably transfected with pBABE-*Shox2* and pBABE-Empty (control) during adipocyte differentiation. Data are shown as mean ± SEM of triplicate samples and repeated three times. (*C*) Western blot of *Adrb3* from protein extracts from pBABE-*Shox2* and pBABE-Empty C3H10T1/2 adipocytes after 6 d of differentiation. Western blot for actin was used as a loading control. (*D*) Lipolysis rates after stimulation with isoproterenol as measured by glycerol release from pBABE-Shox2 and pBABE-Empty C3H10T1/2 adipocytes after 8 d of differentiation. Data are graphed as fold induction over basal lipolytic rate ± SEM of three replicates. The entire experiment was repeated twice. (*E*) Lipolysis rates after stimulation with CL 316,243 as measured by glycerol release from pBABE-Shox2 and pBABE-Empty C3H10T1/2 adipocytes after 8 d of differentiation. Data are shown as graphed as fold induction over basal lipolytic rate ± SEM of three replicates. The entire experiment was repeated twice.

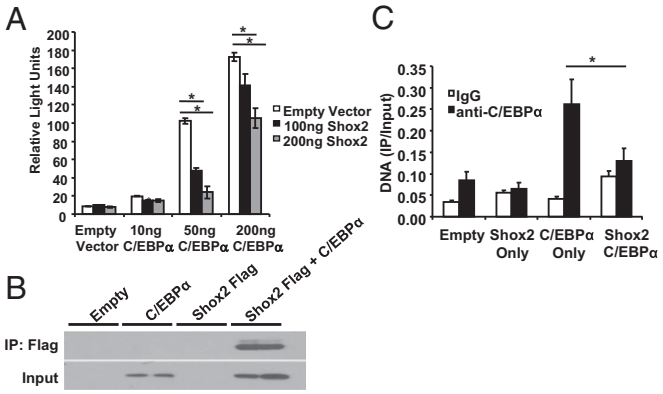

Fig. 6. Shox2 represses C/EBPα transactivation of *Adrb3*-luc constructs. (*A*) Luciferase activity of C3H10T1/2 cells transfected with a 5.13-kb *Adrb3* luciferase reporter and either a vector control or 10, 50, or 200 ng of *C/EBPα* expression vector. C3H10T1/2 cells were also cotransfected with either a vector control or 100 or 200 ng of a *Shox2* expression vector. Data are shown as mean ± SEM of three replicates. The entire experiment was repeated three times. $*P < 0.05$. (*B*) Coimmunoprecipitation experiments performed in C3H10T1/2 cells transiently transfected with C/EBPα alone or in combination with Flag–Shox2. Input and Flag immunoprecipitates (IP) were immunoblotted with C/EBPα-specific antibodies. The entire experiment was repeated twice. (*C*) ChIP Assay for C/EBPα on the Adrb3 promoter in C3H10T1/2 cells after transient transfection with C/EBPα alone or in combination with Flag–Shox2. Data are shown as mean ± SEM of three replicates. The entire experiment was repeated twice.

respectively. Shox2 repressed this induction of *Adrb3*-luc by C/EBPα in a dose-dependent manner by up to 75% (Fig. 6*A*). To determine whether the Shox2 might physically interact with C/EBPα, we transiently transfected C3H10T1/2 cells with vectors expressing C/EBPα and a FLAG-tagged-Shox2. We immunoprecipitated the cell extracts with anti-FLAG and western blotted the precipitates with anti-C/EBPα antibodies. As shown in Fig. 6*B*, Shox2 coimmunoprecipitated with C/EBPα in C3H10T1/2 cells. Thus, Shox2 directly interacts with C/EBPα and represses its activation of the *Adrb3* promoter.

To further assess how Shox2 represses *Adrb3* promoter activity, we performed ChIP analysis on the endogenous *Adrb3* promoter after transiently transfecting C3H10T1/2 cells with vectors expressing C/EBPα and a FLAG-tagged-SHOX2. Interestingly, using an anti-FLAG antibody, we were unable to show recruitment of FLAG-tagged-Shox2, indicating that Shox2 does not bind the Adrb3 promoter. However, the expression of Shox2 interferes with the ability of C/EBPα to bind the *Adrb3* promoter, as C/EBPα recruitment to the *Adrb3* promoter was reduced by about 50% when cotransfected with Shox2 (Fig. 6*C*). Thus, the interaction with Shox2 appears to modulate C/EBPα activity and leads to a decrease in β3-adrenergic receptor expression and decreased lipolysis.

Discussion

The differential physiological effects of visceral versus s.c. obesity and their relative risk of metabolic disease have been documented in multiple studies. Visceral obesity is associated with insulin resistance and metabolic disorders, such as type 2 diabetes, whereas s.c. obesity is not (1, 21). In fact, in some studies, s.c. obesity is even protective against the effects of visceral obesity (3). Several factors have been suggested to contribute to these differences: first, anatomical differences between visceral and s.c. fat, and especially the fact that visceral fat drains directly into the portal vein, exposing the liver to higher levels of free fatty acids, adipokines, and cytokines, which can lead to insulin resistance (22); second, obesity is associated with inflammation and the infiltration of activated macrophages into fat, and these occur to a greater extent in visceral fat compared with s.c. fat (23); and finally, a number of studies indicate intrinsic differences in gene expression between adipocytes from different fat depots, which might lead to different biological and physiological function (6–8).

In the present study, we have explored how one of these differentially expressed genes, *Shox2,* may play an important role in obesity and body fat distribution. We find that *SHOX2* is more highly expressed in s.c. than visceral fat both in mice and humans. Furthermore, *SHOX2* expression in the s.c. fat is significantly higher in both men and women (20% and 40% increased, respectively) with visceral obesity than in individuals with peripheral obesity. Thus, *SHOX2* expression in the s.c. fat is associated with lower ratios of s.c. to visceral fat, i.e., central obesity, suggesting that *SHOX2* may contribute to a differential distribution and function of adipose tissue. Although the modest increase in SHOX2 is unlikely to be solely responsible for the visceral obesity in human subjects, these data fit within a growing body of work that shows that numerous gene pathways contribute to both common obesity and fat distribution (24).

When F-Shox2$^{-/-}$ male mice are place on HFD, they are resistant to diet-induced obesity and show decreased overall fat mass, with greater differences in the s.c. depot. The greater effect on adipose mass in the s.c. versus the peritoneal fat is consistent with the higher levels of *Shox2* in the s.c. fat. This differential distribution of fat is indicated by a change in weight ratio of perigonadal to s.c. fat in male mice made obese by HFD from 1.33 in controls to 2.45 in F-Shox2$^{-/-}$ mice. Thus, although the F-Shox2$^{-/-}$ male mice are protected from HFD-induced obesity, they do this by developing a less favorable fat distribution. Even though F-Shox2$^{-/-}$ male mice on HFD are less obese than controls, they do not exhibit the beneficial effects of reduced obesity on metabolic parameters such as glucose tolerance and insulin levels. These data are consistent with previous studies in rodents and humans (2–4, 25) indicating that, in contrast to visceral fat, which has detrimental metabolic effects, s.c. fat may have a beneficial effect on metabolism.

The major contributing factors to this differential effect on adipose depots appear to be differential changes in lipolytic and lipogenic rates. The effect of Shox2 ablation on the growth of the s.c. and visceral depots is relative to the levels of Shox2 in these fat pads. The lipolysis rates of adipocytes from the s.c. and, to a lesser extent, perigonadal depots are increased in F-Shox2$^{-/-}$ males compared with controls. In perigonadal adipocytes, this increased rate of lipolysis is coupled with increased insulin-stimulated lipogenesis, and these opposing factors almost balance each other, leading to only a modest reduction of perigonadal tissue mass. On the other hand, in s.c. adipocytes, the higher rates of lipolysis are not coupled to higher lipogenic rates, leading to a reduction in lipid storage and the reduced adipose cell size observed in the s.c. fat. The knockdown of *Shox2* in C3H10T1/2 cells also leads to a decrease of lipid accumulation under basal conditions, suggesting that Shox2 also impacts some other aspect of adipocyte biology, such as lipid storage. However, the mechanism behind these effects is unclear, as *Shox2* ablation did not change the expression of regulators of lipid storage.

The increased isoproterenol-stimulated lipolysis in adipocytes isolated from HFD-fed F-Shox2$^{-/-}$ male mice can be explained by an increase in β3-adrenergic receptor, especially in the s.c. fat. These data are consistent with previous studies that have shown that, in murine adipose tissue, lipolysis is regulated primarily by Adrb3 activation (26) and that inactivation of *Adrb3* leads to an increase of fat stores that is accentuated by high fat feeding (18). The phenotype that we observe in F-Shox2$^{-/-}$ mice is due to an increase in Adrb3, an increase in lipolysis, and a decrease in fat accumulation on HFD. These changes are the opposite of those observed in the *Adrb3* knockout mice. Other physiological regulators of Adrb3 have recently been studied. For example, ablation of SERTA domain containing 2 (TRIP-Br2), similar to

Shox2 ablation, leads to increase in Adrb3 and an increase in the rate of catecholamine-induced lipolysis. However, unlike inactivation of Shox2, TRIP-Br2 also leads to changes in oxidative gene metabolism and thermogenesis (27). Although F-Shox2$^{-/-}$ mice exhibit resistance to diet-induced obesity, unlike TRIP-Br2 knockout mice, they exhibit higher levels of circulating FFA and do not have improved insulin resistance. Together, these results suggest that, to improve metabolic parameters, free fatty acids generated through lipolysis must be used in situ by the adipocyte, and not released into the circulation.

No effect of Shox2 alone was observed on *Adrb3* transcription. However, the *Adrb3* promoter is directly transactivated by C/EBPα, a transcription factor that rises markedly during adipocyte differentiation, and Shox2 is able to able to repress this activation in a dose-dependent manner by directly interacting with C/EBPα and reducing C/EBPα binding to the *Adrb3* promoter. Interestingly, *Shox2* deficiency or overexpression does not change the expression of other target genes of C/EBPα during adipogenesis, such as *PPARγ* and *aP2*. However, recent studies have demonstrated that attenuation of C/EBP signaling reduces Adrb3 action without changes in other C/EBPα target genes (28). *Adrb3* may act as a sensitive barometer of C/EBPα activity because it is not further activated by PPARγ (29).

In conclusion, in this study, we demonstrate that *Shox2* plays a significant role in determining adipose distribution in both humans and mice. In human subjects, the expression level of *SHOX2* in the s.c. fat is positively correlated with visceral obesity. Specific ablation of *Shox2* from mouse adipocytes attenuates diet-induced obesity by reducing adipocyte size, especially in s.c. fat, where SHOX2 levels are normally high. This decrease in adipocytes size is secondary to greater rates of lipolysis due to an increase in *Adrb3* expression. Studies in cell culture models further demonstrate that Shox2 controls lipolytic rate and *Adrb3* expression in a dose-dependent manner. Taken together, these data indicate that Shox2 serves as an important modulator of adipocyte function, and its differential expression contributes to the depot-specific differences in adipocyte behavior.

Materials and Methods

Further details are presented in *SI Materials and Methods*. The generation of F-Shox2$^{-/-}$ mice has already been described (16). mRNA and protein expression was measured by qPCR and western blot, respectively. Lipogenesis was measured by incorporation of [^{14}C] D-glucose into lipids, and lipolytic activity measured by glycerol release. Shox2 was stably overexpressed by retroviral transduction, and stably knocked down by lentiviral transduction.

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