Tissue-specific disruption of Kbtbd2 uncovers adipocyte-intrinsic and -extrinsic features of the teeny lipodystrophy syndrome

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Loss of KBTBD2 in all tissues causes the teeny phenotype, characterized by insulin resistance with late failure of insulin production, severe hyperglycemia/diabetes, lipodystrophy, hepatosteatosis, and growth retardation. KBTBD2 maintains insulin sensitivity in adipocytes by restricting the abundance of p85α. However, the possible physiological contribution or contributions of KBTBD2 have not yet been examined in other tissues. Here we show that mice with an adipocyte-specific knockout of Kbtbd2 accumulate p85α in white and brown adipose tissues, causing insulin resistance, moderate rather than severe hyperglycemia, sustained hyperinsulinemia without late failure of insulin production, and lipodystrophy leading to ectopic lipid accumulation in the liver. Adipocyte-extrinsic insulin resistance was observed in liver and muscle. None of these abnormalities were observed in liver- or muscle-specific Kbtbd2 knockout mice. Mice with Kbtbd2 knockout in adipocytes, liver, and muscle all showed normal growth, suggesting that KBTBD2 may be necessary to ensure IGF1 signaling in other tissues, notably bone. While much of the teeny phenotype results from loss of KBTBD2 in adipocytes, some features are adipocyte-extrinsic.

KBTBD2 | p85α | adipocytes | diabetes | insulin resistance

Insulin signaling is essential for the development and function of adipose tissue (1). Insulin drives the differentiation of pre-adipocytes to adipocytes by allowing glucose uptake and de novo triglyceride synthesis. Defects in differentiation of adipocytes or triglyceride synthesis cause a near-complete absence of adipose tissue, termed lipodystrophy. The binding of insulin to the insulin receptor (IR) activates the IR β subunit, which phosphorylates IR substrates IRS1 and IRS2, causing the activation of the phosphatidylinositol 3-kinase (PI3K) and the downstream AKT pathway (2), which, among other things, permits the up-regulation of glucose transporters on the surface of insulin-sensitive cells. Somewhat paradoxically, lipodystrophy can also be caused by tissue-specific insulin resistance, which can itself cause generalized insulin resistance and diabetes.

Recently we detected a phenotype termed teeny among third-generation C57BL/6J mice homozygous for N-ethyl-N-nitrosourea-induced mutations (3). The teeny phenotype is characterized by growth retardation, lipodystrophy, extreme insulin resistance, severe diabetes (with a fasting glucose level as high as 600 to 700 mg/dL), and hepatosteatosis. The causative mutation created a premature stop codon within Kbtbd2, which encodes a Kelch repeat and BTB (POZ)-domain containing protein. KBTBD2 functions together with CULLIN3 (CUL3) as a E3 ubiquitin ligase complex targeting p85α, the regulatory subunit of PI3K (3). Loss of KBTBD2 leads to a 30-fold accumulation of p85α in adipocytes (3). The excess monomeric p85α inhibits the insulin signaling pathway through competition with p85α-p110 for binding to IRS1 (3) and formation of a sequestration complex between p85α and IRS1 (4). Loss of KBTBD2 causes lipodystrophy, suggesting the importance of KBTBD2 in adipose tissue development. However, although transplantation studies suggested that some aspects of the teeny phenotype were adipocyte-intrinsic (3), expression of Kbtbd2 mRNA is observed in other insulin-responsive tissues (e.g., muscle and liver) that are less readily evaluated through transplantation. Loss of KBTBD2 from these tissues might contribute to the teeny phenotype as well.

To determine the essential functions of KBTBD2 in specific tissues, we generated conditional knockout alleles activated by cre recombinase in three major insulin responsive tissues: adipose tissue, muscle, and liver. We find that loss of KBTBD2 in adipocytes is sufficient to cause lipodystrophy, hepatosteatosis, insulin resistance, and hyperglycemia. However, hyperglycemia was less severe than that observed in the teeny homozygotes, and late failure of insulin production was not observed, as it was in teeny homozygotes. Isolated conditional knockouts of Kbtbd2 in adipose, muscle, or liver tissues was not sufficient to cause growth retardation, which remains unexplained.

Results

Generation of Kbtbd2 Conditional Knockout Mice. To study the role of KBTBD2 in different tissues, we used CRISPR/Cas9-mediated gene editing to create a conditional knockout allele of Kbtbd2 with exon 3 (Ex3) flanked by LoxP sites (Kbtbd2-flx) (Fig. 1A). Ex3 of Kbtbd2 contains a 166-bp sequence encoding amino acids 57 to 112 of the 623-aa KBTBD2 protein. The removal of Ex3 caused a deletion and frameshift, resulting in a null allele (Fig. 1A). Two different CRISPR sgRNAs (#1, #2) that targeted the left and right flanking introns of Ex3 were co-injected with two 200-μg oligo DNA templates into C57BL/6J

Significance

To examine the function of KBTBD2 in different cell types, we injected the gene out in adipocytes, liver, and muscle cells. None of these conditional knockouts could reproduce the growth retardation observed in mice with a global knockout. An adipose tissue-specific knockout was similar to a global knockout in most respects; however, hyperglycemia was less severe and hyperinsulinemia was more sustained. An adipose tissue-specific knockout caused insulin resistance in other tissues, demonstrating an adipose-extrinsic effect of the mutation.

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zygotes (Fig. 1A). The Kbtbd2-floxed mice were crossed to different promoter-driven cre-recombinase transgenic mice to disrupt Kbtbd2 in specific tissues. Adiponectin promoter-driven cre-recombinase (Apn-cre) (5) caused a specific deletion of Ex3 in interscapular brown adipose tissue (iBAT), epididymal white adipose tissue (eWAT), and inguinal white adipose tissue (iWAT; Fig. 1B). Myogenin promoter-driven cre-recombinase (Myog-cre) (6) caused a specific deletion of Ex3 in skeletal muscle (Fig. 1B). Albumin promoter-driven cre-recombinase (Alb-cre) (7) caused a specific deletion of Ex3 in liver (Fig. 1B). After successful generation of these tissue-specific Kbtbd2 knockout models, we examined the accumulation of p85α, which is the direct target of KBTBD2 E3 ubiquitin ligase (3), in each of the three tissues. Loss of KBTBD2 in adipocytes caused a dramatic accumulation of p85α in all three adipose tissues tested (iBAT, eWAT, and iWAT), with no obvious change of p85α levels in liver and muscle (Fig. 1C). We separated adipose tissue into floating adipocytes and the stromal vascular fraction (SVF) (SI Appendix, Fig. S1A) and observed a specific accumulation of p85α in adipocytes, but not in the SVF (SI Appendix, Fig. S1B). Loss of KBTBD2 in skeletal muscle did not change the level of p85α in muscle, adipose tissues, or liver (Fig. 1C). In liver-specific Kbtbd2 knockout mice, there was a slight increase in liver p85α, but not in adipose or muscle tissues (Fig. 1C). These data suggest cell-intrinsic degradation of p85α by KBTBD2 in adipocytes, and to a much lesser extent, hepatocytes.

**Loss of KBTBD2 in Adipocytes Causes Lipodystrophy, but Not Growth Retardation.** Growth retardation was observed in teeny and Kbtbd2−/− mice within the first few days and throughout postnatal life (3). We first examined whether any of the tissue-specific Kbtbd2 knockouts contribute to the growth defect. In contrast to 8-wk-old Kbtbd2−/− mice, loss of Kbtbd2 in adipose tissue, muscle, or liver did not affect the body weight (Fig. 2A) or body length (Fig. 2B), suggesting that KBTBD2 may affect growth through its function in other tissue or tissues.

A congenital systemic Kbtbd2 knockout causes severe lipodystrophy (3), but it is not known whether the lipodystrophy is caused by the function of KBTBD2 in adipose tissue, muscle, liver, or a combination of these tissues. At 8 wk of age, MRI showed that only adipocyte-specific Kbtbd2 knockout mice were as lean as Kbtbd2−/− mice (Fig. 2C and D and SI Appendix, Fig. S2). No changes in fat weight or lean weight were observed in muscle-specific Kbtbd2 knockout mice. Although the fat weight of liver-specific Kbtbd2 knockout mice was slightly increased, this difference did not reach statistical significance (Fig. 2C and SI Appendix, Fig. S2A). Consistent with the body composition result, adipocyte-specific Kbtbd2 knockout mice had reduced leptin and adiponectin in the serum, presumably as a consequence of fat loss (Fig. 2E and F). No changes in leptin and adiponectin were observed in muscle-specific Kbtbd2 knockout mice (Fig. 2E and F). We also observed slightly increased leptin and adiponectin levels in liver-specific Kbtbd2 knockout mice (Fig. 2E and F), although similar to the change in fat mass measured by MRI, these changes were not statistically significant.

Necropsy revealed that both eWAT and iWAT of 16-wk-old adipocyte-specific Kbtbd2 knockout mice were reduced in size compared with Kbtbd2flox/flox littermates (Fig. 2H and I), with only a slight reduction in iBAT (Fig. 2G). Hematoxylin and eosin (H&E) staining revealed slightly irregular adipocytes in iBAT of
Fig. 2. Adipocyte-specific Kbtbd2 knockout mice develop lipodystrophy despite having a normal growth rate. (A–D) Body weight (A), body length (B), fat weight (C), and lean weight (D) of 8-wk-old male mice. (E–F) Serum leptin (E) and adiponectin (F) of 8-wk-old male mice after a 6-h fast. (G–I) Representative photographs of iBAT (G), eWAT (H), and IWAT (I) from 16-wk-old male mice. (J–O) H&E staining of sections from different adipose tissues of 16-wk-old male mice. (Scale bar: 100 μm.) Data are representative of two independent experiments (A–I) or one experiment (J–O). Data points represent individual mice (A–F). Data are presented as means ± SEM (A–F). P values were determined by one-way ANOVA with Tukey's multiple comparison test. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001; ns, not significant with P > 0.05.
16-wk-old adipocyte-specific Kbtbd2 knockout mice (Fig. 2 J and K). The eWAT and iWAT of these mice had irregular adipocytes with infiltration of nonfat cells (Fig. 2 L–O). We also noticed a larger variation in the size of adipocytes in both eWAT and iWAT of adipocyte-specific Kbtbd2 knockout mice, suggesting the heterogeneity of adipocytes within adipose tissues. These data demonstrate that KBTBD2 functions directly in adipocytes either to maintain the mass of adipose tissues or is developmentally important for the generation of adipose tissue.

**Loss of KBTBD2 in Adipocytes Causes Hepatosteatosis.** Hepatosteatosis is another phenotype observed in Kbtbd2 knockout mice (3). We utilized different Kbtbd2 tissue-specific knockout mice to explore the exact role of KBTBD2 deficiency in the development of hepatosteatosis. Despite the high expression of Kbtbd2 mRNA and protein in liver (3), knockout of Kbtbd2 in adipocytes caused large and pallid fatty livers in 16-wk-old mice, but knockout in the liver or muscle did not (Fig. 3 A–D). Similarly, knockout of Kbtbd2 only in adipocytes increased levels in serum alanine aminotransferase (ALT), which is an indication of liver damage (Fig. 3 E). Histological analysis of the livers by H&E and Oil Red O staining likewise showed that only knockout of Kbtbd2 in adipocytes caused increased lipid accumulation in the tissue (Fig. 3 F–M). Taken together, these findings clearly indicate that the hepatosteatosis in Kbtbd2 knockout mice is a consequence of the loss of KBTBD2 in adipocytes. This is consistent with the frequently observed hepatic steatosis characteristic of most partial lipodystrophies. Notably, hepatosteatosis was not observed in mice with conditional knockouts of Kbtbd2 in liver or muscle.

**The Role of KBTBD2 in Glucose Metabolism and Insulin Sensitivity.** Adipose tissue, liver, and muscle are insulin-responsive tissues. We used Kbtbd2 tissue-specific knockout mice to directly determine whether systemic insulin sensitivity was dependent on KBTBD2 in adipose, muscle, or liver tissue, and whether the dependence was cell-intrinsic in each case. Only adipocyte-specific Kbtbd2 knockout mice had elevated fasting glucose and insulin levels (Fig. 4 A and B). In contrast to Kbtbd2+/− mice, which have a persistent high fasting glucose level around 600 to 700 mg/dL (Fig. 4 A and SI Appendix, Fig. S3A, and ref. 3), adipocyte-specific Kbtbd2 knockout mice had a significantly smaller increase in fasting glucose, which reached around 300 to 400 mg/dL (Fig. 4 A and SI Appendix, Fig. S3A). The increased fasting insulin level was similar to that observed in Kbtbd2−/− mice at 8 wk of age (Fig. 4 B). However, we did not observe a decline in insulin levels in adipocyte-specific Kbtbd2 knockout mice at 16 wk of age, as we previously observed in Kbtbd2−/− mice (SI Appendix, Fig. S3A and ref. 3). Insulin tolerance tests showed dramatic whole-body insulin resistance in adipocyte-specific Kbtbd2 knockout mice, but not in muscle- or liver-specific Kbtbd2 knockout mice (Fig. 4 C–E). We also tested the insulin sensitivity of eWAT, muscle, and liver by examining insulin-stimulated AKT phosphorylation at Ser-473 (p-AKT) (473). Adipocyte-specific Kbtbd2 knockout mice had impaired insulin-stimulated p-AKT in eWAT, as well as in muscle and liver (Fig. 4 F). We did not observe a change in insulin-stimulated p-AKT in muscle- and liver-specific Kbtbd2 knockout mice (Fig. 4 G and H). These data suggest that KBTBD2 in adipocytes is critical for maintaining glucose metabolism and insulin sensitivity, and that loss of KBTBD2 in adipocytes also causes insulin resistance in liver and muscle.

![Figure 3](#fn1)

**Fig. 3.** Loss of Kbtbd2 in adipocytes causes liver triglyceride accumulation and damage. (A–C) Representative photographs of liver from 16-wk-old male mice. (D–F) Liver triglyceride (D) and serum ALT (E) in 16-wk-old male mice. (F–M) Liver sections of 16-wk-old male mice stained with H&E (F–I) and Oil Red O (J–M). (Scale bar: 100 μm.) Data are representative of two independent experiments (A–E) or one experiment (F–M). Data points represent individual mice (D–E). Data are presented as means ± SEM (D–E). P values were determined by one-way ANOVA with Tukey’s multiple comparison test. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001; ns, not significant with P > 0.05.
Discussion

In our previous work, the transplantation of WT adipocytes to teney mice of postweaning age was shown to rescue hyperglycemia, insulin resistance, and hepatosteatosis, while failing to correct body length (3). This suggested that KBTBD2 deficiency in adipocytes accounted for most of the teney phenotype, but did not exclude a contribution by other tissues. It was, in fact, notable that total body insulin resistance was fully alleviated by adipose tissue transplantation, given the supposition that insulin signaling in muscle and liver cells, collectively representing a large proportion of total body weight, might still be abnormal. As to the failure of adipocyte transplantation to rescue the shortfall in body length, we reasoned that intervention might have come too late to do so.

To more directly examine the essential functions of KBTBD2 in insulin signaling in other tissues, and to determine whether adipose, muscle, or liver tissue KBTBD2 activity might be independently required for growth, we made use of tissue-specific conditional Kbtbd2 knockout mutations in the present study. We have also been able to assess the effects of KBTBD2 loss from each of these tissues on the physiology of the other two tissues.

Fig. 4. The regulation of insulin sensitivity by KBTBD2 in adipose tissue, liver, and muscle. (A and B) Blood glucose (A) and serum insulin (B) in 8-wk-old male mice after a 6-h fast. (C–E) Insulin tolerance test. Blood glucose was measured at indicated times after i.p. insulin injection in 12-wk-old male mice [(C) n = 8 Kbtbd2flox/flox, 6 Kbtbd2flox/flox; Alb-cre, (D) n = 6 Kbtbd2flox/flox, 3 Kbtbd2flox/flox; Myog-cre, (E) n = 8 Kbtbd2flox/flox, 5 Kbtbd2flox/flox; Alb-cre]. (F and G) Immunoblots of different tissue lysates of 12-wk-old male Kbtbd2flox/flox mice and Kbtbd2flox/flox littermates 30 min after control or insulin injection. Data are representative of two independent experiments. Data points represent individual mice (A and B). Data are presented as means ± SEM (A–E). P values were determined by one-way ANOVA with Tukey’s multiple comparison test (A and B) or Student’s t test (C–E). *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001; ns, not significant with P > 0.05.
Loss of KBTBD2 in adipocytes causes striking accumulation of p85α in brown and white adipose tissues, and leads to diminution in the quantity of these adipose tissues, as observed in teeny mice. It also caused insulin resistance and hyperglycemia. This led to secondary hepatosteatosis, as well as insulin resistance in both liver and muscle cells. In contrast, in the liver-specific Kbtbd2 knockout mice, we only observed a slightly increased level of p85α, and no significant effect on adipose tissue mass. In the muscle-specific Kbtbd2 knockout mice, no change at all was observed in the level of p85α, and no effect on adipose tissue mass was observed. Insulin resistance was not evident in any of the three tissues examined, nor systemically. It would appear that when adipocytes exhibit severe insulin resistance and are few in number, as in teeny mice or adipocyte-specific Kbtbd2 knockout mice, excess lipid spills over into the liver. But the converse situation is not observed when KBTBD2 is absent in the liver or muscle; in each case, there is no detectable effect on cell-intrinsic insulin sensitivity, nor on adipose tissue mass. 

Adipocyte-specific Kbtbd2 knockout mice display a reduction of fat mass versus lean body mass, as well as p85α accumulation similar to Kbtbd2−/− mice. We also observed a comparable level of liver triglyceride accumulation and liver damage in these mouse models. Despite these similarities, the extent of hyperglycemia and the changes in insulin level are different. Kbtbd2−/− mice have a fasting blood glucose of around 600 to 700 mg/dL, while adipocyte-specific Kbtbd2 knockout mice have a fasting blood glucose of around 300 to 400 mg/dL and no glycosuria even in the fed state. The fasting blood insulin levels of Kbtbd2−/− mice increases over time and peaks around 7 wk of age, then declines to near WT levels (3). The fasting insulin level in adipocyte-specific Kbtbd2 knockout mice remains high at least through 16 wk of age. Pancreatic β cells respond to systemic insulin resistance with increased insulin secretion, which entails expansion of β-cell mass and enhanced β-cell function (8). The rapid reduction of insulin levels in Kbtbd2−/− mice beginning at 7 wk of age suggests that exhausted β cells are unable to further produce insulin to decrease the high level of blood glucose. However, the adipocyte-specific Kbtbd2 knockout mice maintain ample β-cell function until at least 16 wk of age. It is possible that the phenotypic effect of global Kbtbd2 knockout is more severe than conditional Kbtbd2 knockout in adipose tissues due to additional effects of KBTBD2 disorders in individual tissues. Alternatively, these differences may be a result of KBTBD2 deficiency within β cells themselves.

The failure of adipocyte-specific knockout of Kbtbd2 to recapitulate the growth retardation observed in teeny mice might be attributable to the importance of KBTBD2 in tissues yet to be examined, and specifically in the preservation of insulin-like growth factor 1 (IGF1) signaling. IGF1 is produced largely by the liver under the influence of pituitary growth hormone and strongly stimulates the growth of most tissues, including bones. IGF1 signaling engages the same PI3K–AKT axis as insulin to elicit biological responses (9). We have earlier observed that IGF1 levels are dramatically elevated in Kbtbd2−/− mice (3). We interpret this as a homeostatic accommodation to growth failure and the metabolic changes that accompany it. We further suggest that p85α accumulation interrupts signaling from the IGF1 receptor (IGF1R), just as it interrupts signaling from the insulin receptor. We observed normal body weight and length in mice with isolated deletions of Kbtbd2 in adipocytes, liver, and muscle (although not in Kbtbd2−/− mice). It would be informative to target Kbtbd2 specifically in bone or cartilage.

Among the advantages of the tissue-specific conditional knockout approach used here, we are able to identify cell-intrinsic and cell-extrinsic effects of gene deletion in a system that is hormonally interconnected. As we have now shown, adipocyte-specific knockout of Kbtbd2 causes marked systemic insulin resistance. While this is initiated in adipocytes, wherein the insulin signal is interrupted by an excess of p85α, it does not remain restricted to adipocytes. Although genetically normal, muscle and liver also become insulin resistant, as revealed by their failure to phosphorylate AKT at residue S473. It remains to be determined whether resolution of this lesion, either by fat transplantation or by genetic reversion, or by attenuation of insulin production, would reverse insulin resistance in other tissues, and if so, over what time frame. The pathogenesis of insulin resistance has been ascribed to various mechanisms, including ectopic lipid accumulation, unfolded protein response pathway activation, and inflammation (10). Activation of protein kinase C isoforms by hepatic diacylglycerol, as well as increased ceramide levels, have also been proposed to impair insulin signaling (11). These events may eventually lead to modification of key insulin signaling proteins, such as serine/threonine phosphorylation of IRS1 (12). Our adipocyte-specific Kbtbd2 knockout mice provide a system with which to further explore secondary insulin resistance mechanisms. In any case, it is clear that in mice with an adipocyte-specific Kbtbd2 knockout, primary insulin resistance in adipocytes becomes generalized through the induction of secondary insulin resistance in both liver and muscle, although neither of these tissues is genetically abnormal. The effects of the adipocyte-specific knockout that we report here could be caused either by the lack of KBTBD2 in mature adipocytes or by a developmental phenotype that prevents adipogenesis from properly occurring.

Kbtbd2 mRNA can be detected in many mouse tissues (3). Despite the presence of both KBTBD2 and p85α in adipose tissues, liver, and muscle, physiologically significant degradation of p85α by KBTBD2 has so far been observed only in adipose tissues. It appears that KBTBD2 becomes necessary for restriction of p85α in mature adipocytes based on the following two observations: in adipocyte-specific Kbtbd2 knockout mice, p85α only accumulates in isolated floating fat cells, remaining unchanged in the SVF, which contains preadipocytes and immune cells, and mouse embryonic fibroblasts isolated from Kbtbd2−/− mice have similar levels of p85α, but p85α only accumulates in mouse embryonic fibroblasts after they are differentiated into adipocytes (3). Further investigation will be needed to understand how and why KBTBD2 differentially degrades p85α in different cells and tissues.

Materials and Methods

Mice. C57BL/6J mice (stock#000664), Apn-cre mice [B6.FVB-Tg(Adipoq-cre)1Evdjl, stock#028020], and Alb-cre mice [B6.129S7-Gpber6-ps1Tg(Alb-cre)Mgn/J, stock#003574] were purchased from The Jackson Laboratory. The Myog-cre mice (6) were provided by Eric Olson (University of Texas Southwestern Medical Center). Kbtbd2 knockout mice (C57BL/6J-Kbtbd2−/−) were generated in our laboratory, as previously described (3). Kbtbd2-flx/fox mice (C57BL/6J-Kbtbd2flx/flx) were generated in our laboratory, using CRISPR/Cas9-mediated gene replacement with the following sgRNAs and oligo templates:

- sgRNA#1, 5′-CCGCAGTGGCAAATAGTAGC-3′
- sgRNA#2, 5′-TGAGTGGTGACGTACTCGGC-3′
- Oligo#1, 5′-GGGCTCTTTTTAAAAGAAGATCTAATCTTGGTTTGCTGCTGAAGCAGGATGCGAGCAGCTATCTTGTTTAATTAAATTAAATATGGAAGAAGCTGGATGACATCTCTGAGATTAACATGGAAATGAAACTCGATTTC-3′
- Oligo#2, 5′-CAGAGCTGACCTTGAATCTTGTTGTGTTATCGAGCAGAAGAATATTAAATGAAACTCGATTTC-3′.

Tissue-specific knockout of Kbtbd2 was achieved by breeding Kbtbd2-flx/fox mice with mice expressing each cre construct. All mice were fed standard chow diet (2016 Teklad Global 16% Protein Rodent Diet) and housed at room temperature (22° C). Mice were maintained at the University of Texas Southwestern Medical Center.
of Texas Southwestern Medical Center, and studies were performed in accordance with institutionally approved protocols. All experiments in this study were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee.

Metabolic Analysis. Mice were fasted for 6 h (7:00 AM to 1:00 PM) for insulin tolerance tests. Blood glucose was tested with the AlphaTRAK glucometer and test strips. After the first blood glucose measurement, the insulin tolerance test was initiated by i.p. injection with human insulin (0.75 U/kg; Sigma-Aldrich) and blood glucose was measured at set times during the next 2 h. For insulin-stimulated AKT activation, insulin or saline was injected i.p. at a dose of 1.5 U/kg body weight after a 6-h fast. Tissues were collected at 30 min postinjection.

Blood/Serum Chemistries, ELISA, Liver Triglyceride Measurement, and Immunohistochemistry. Mice were fasted for 6 h (7:00 AM to 1:00 PM) for all blood sample collections. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure insulin (Crystal Chem) and leptin (Crystal Chem) in the serum, according to the manufacturer’s instructions. Serum ALT and liver triglyceride were measured by the University of Texas Southwestern Medical Center Metabolic Phenotyping Core with standard protocols. Samples for histology were harvested from anesthetized mice and fixed according to standard procedures. The staining protocols for H&E staining and Oil Red O staining were described previously (3).

Sample Preparation, DNA Agarose Gel, and Western Blot Analysis. Tissue samples were snap frozen in liquid nitrogen immediately after dissection. Isolation and separation of murine primary adipocytes and SVF cells from iWAT were performed as described previously (13). TRIzol (Invitrogen) reagent was used to isolate genomic DNA and protein from the same sample in a sequential manner following a standard protocol. Genomic DNA was amplified with primers flanking Ex3 and two LoxP sites to detect the Ex3 deletion with 1.5% DNA agarose gel. Protein samples were normalized to 4 μg/mL in 1× NuPAGE lithium dodecyl sulfate sample buffer (Life Technologies) with 2.5% (vol/vol) 2-mercaptoethanol (Sigma-Aldrich). For western blots, 5-μL samples (20 μg total protein) were resolved by NuPAGE 4% to 12% (wt/vol) Bis-Tris gels (Thermo Fisher Scientific), transferred to nitrocellulose membranes (Bio-Rad), blotted with the primary antibody at 4 °C overnight and the secondary antibody for 1 h at room temperature, and then visualized by chemiluminescent substrate (Thermo Fisher Scientific). The following primary antibodies were used in this study: rabbit anti-p85α, anti-Gapdh, and anti-pAKT^{S473} (Cell Signaling Technology).

Data Availability. All data generated in this study are included in this published article and the SI Appendix.

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