Brown adipose expansion and remission of glycemic dysfunction in obese SM/J mice

Caryn Carson  
*Washington University School of Medicine in St. Louis*

Juan F Macias-Velasco  
*Washington University School of Medicine in St. Louis*

Subhadra Gunawardana  
*Washington University School of Medicine in St. Louis*

Mario A Miranda  
*Washington University School of Medicine in St. Louis*

Sakura Oyama  
*Washington University School of Medicine in St. Louis*

See next page for additional authors
Brown Adipose Expansion and Remission of Glycemic Dysfunction in Obese SM/J Mice

Graphical Abstract

Highlights

- High-fat-fed SM/J mice develop disproportionate expansion of brown adipose
- The increased brown adipose improves insulin-stimulated glucose uptake
- Expanded brown adipose has a healthier transcriptional profile and lower inflammation
- Sfrp1 expression correlates with brown adipogenesis and glucose homeostasis

Authors
Caryn Carson, Juan F. Macias-Velasco, Subhadra Gunawardana, ..., Heather Schmidt, Jessica P. Wayhart, Heather A. Lawson

Correspondence
lawson@wustl.edu

In Brief
Carson et al. characterize the spontaneous development of a disproportionate expansion of brown adipose tissue in obese SM/J mice. They find the mice with increased brown adipose tissue have improved glucose tolerance and insulin sensitivity without weight loss, and identify the cytokine Sfrp1 as a likely cause of brown adipogenesis.
SUMMARY

We leverage the SM/J mouse to understand glycemic control in obesity. High-fat-fed SM/J mice initially develop poor glucose homeostasis relative to controls. Strikingly, their glycemic dysfunction resolves by 30 weeks of age despite persistent obesity. The mice dramatically expand their brown adipose depots as they resolve glycemic dysfunction. This occurs naturally and spontaneously on a high-fat diet, with no temperature or genetic manipulation. Removal of the brown adipose depot impairs insulin sensitivity, indicating that the expanded tissue is functioning as an insulin-stimulated glucose sink. We describe morphological, physiological, and transcriptomic changes that occur during the brown adipose expansion and remission of glycemic dysfunction, and focus on Sfrp1 (secreted frizzled-related protein 1) as a compelling candidate that may underlie this phenomenon. Understanding how the expanded brown adipose contributes to glycemic control in SM/J mice will open the door for innovative therapies aimed at improving metabolic complications in obesity.

INTRODUCTION

An estimated 10%–30% of obese individuals maintain glycemic control, and some longitudinal studies suggest their risk of developing type II diabetes is no greater than matched lean individuals (Meigs et al., 2006). No causative factors underlying glycemic control in obesity have been discovered; however, the strongest predictors of impaired glycemic control in obesity are increased visceral fat mass and adipose tissue dysfunction (Goossens, 2017; Küting et al., 2010). Thus, research efforts have focused on understanding the genetic and physiological mechanisms of action of adipose. Recent research reveals that brown adipose activity is associated with anti-diabetic properties. Cold exposure in both obese and lean individuals causes increased uptake of fatty acids and glucose into brown adipose tissue (Saito et al., 2009). Further, increased brown adipose activity has been shown to improve glucose homeostasis and insulin sensitivity in adults (Chondronikola et al., 2014). Transplantation of brown adipose tissue into obese mice greatly improves glucose parameters, including fasting glucose levels and response to a glucose challenge (Gunawardana and Piston, 2012). Although there are a variety of obese and diabetic mouse models, there are no mouse models for understanding the relationship between brown adipose and glycemic control in obesity.

The SM/J inbred mouse strain has long been used for studying interactions between diet and metabolism, and more recently has helped uncover the genetic architecture underlying diet-induced obesity and glucose homeostasis. It has previously been shown that fed a high-fat diet, SM/J mice display many of the characteristics of a diabetic-obese mouse: obesity, hyperglycemia, and glucose intolerance at 20 weeks of age (Cheverud et al., 2011; Ehrich et al., 2003; Lawson et al., 2010, 2011). We discovered that SM/J mice undergo a remarkable transformation between 20 and 30 weeks of age. Despite persistence of the obese state, these mice enter into diabetic remission: returning to normoglycemia and reestablishing glucose tolerance and improving insulin sensitivity. Contemporary with this remission of glycemic parameters is a dramatic expansion of the intrascapular brown adipose depot. Our results strongly suggest that SM/J’s brown adipose contributes to their diabetic remission through non-thermogenic mechanisms, and we focus on the cytokine Secreted Frizzled-Related Protein 1 (SFRP1) as a compelling candidate that may underlie this remarkable phenomenon. This study describes the morphological, physiological, and transcriptomic changes that occur during this transition, and establishes the SM/J mouse as a unique model for understanding the relationship between brown adipose and glycemic control in obesity. Understanding this relationship in a genetic model of glycemic resolution will set the stage for identifying novel, potentially therapeutic targets for the improvement of glycemic control.

RESULTS

SM/J Mice Improve Glucose Parameters without Weight Loss

When fed a high-fat diet (Table S1) from 3 weeks of age, SM/J mice develop obesity, hyperglycemia, and impaired glucose tolerance by 20 weeks (Ehrich et al., 2003). By 30 weeks, despite

the persistence of obesity, high-fat-fed SM/J mice resolve their hyperglycemia and impaired glucose tolerance to levels indistinguishable from low-fat-fed controls (Figures 1A–1D). Thirty-week high-fat-fed SM/J mice have dramatically increased serum and pancreatic insulin levels compared with 20-week animals (Miranda et al., 2020), and insulin sensitivity improves along with improved glycemic parameters (Figures 1E and 1F). Notably, metabolic hormones, such as adiponectin, glucagon, IGF1, and leptin, do not show any changes in circulating levels between 20 and 30 weeks (Figures S1 A–S1D). Further, the changes in glucose phenotypes are not transient effects; rather, high-fat-fed animals maintain improved basal glucose, glucose tolerance, and insulin tolerance as they age (Figures S1 E–S1I).

High-Fat-Fed SM/J Mice Expand Their Interscapular Brown Adipose Tissue Depots

Contemporary with the resolution of glycemic parameters, high-fat-fed SM/J mice dramatically expand their intrascapular brown adipose depots, which is not seen in the LG/J strain of mice on the same diets at any age (Figure S1 K). To understand whether the tissue mass expansion is due to increased size of individual cells or to increased number of total cells, we quantified adipocyte cell size and the mitotic index. There are no significant differences in average cell size in high-fat-fed mice between 20 and 30 weeks or relative to low-fat-fed controls (Figure S2A). Mice on both diets undergo altered adipocyte area profiles between 20 and 30 weeks of age; however, the low-fat tissue develops a profile significantly trending toward larger adipocytes at 30 weeks ($p = 6.4 \times 10^{-7}$), whereas the high-fat tissue develops a profile significantly trending toward smaller adipocytes at 30 weeks ($p = 2.2 \times 10^{-16}$) (Figures 2D and 2E). This suggests that the expansion of the brown adipose depot in high-fat-fed mice is not the result of increased lipid uptake into already existing adipocytes. Quantification of brown adipose cells stained positive for the mitotic marker phosphohistone H3 showed a trend toward a higher mitotic index in the brown adipose of high-fat-fed animals (Figure S2B), suggesting that the increased mass may be caused by an increased number of cells.

Because obesity has been associated with structural and functional “whitening” of brown adipose depots in rodents...
Figure 2. Brown Adipose Expansion in 30-Week-Old High-Fat-Fed SM/J Mice Is Required for Improved Insulin Tolerance

(A and B) Representative pictures of (A) 20- and (B) 30-week-old high-fat-fed female mice with insets showing larger brown adipose lobes in 30-week animals. 

(C) Quantification of interscapular brown adipose depot as a proportion of total fat mass; n = 16–25 mice per cohort.

(D and E) Cell area density graphs for high-fat- and low-fat-fed cohorts. Data are plotted on a log10 scale for visualization; n = 4 mice per cohort.

(F-H) Fasting glucose (F) and insulin (G) and glucose (H) tolerance were not significantly altered by removal of the brown adipose depot before (20-week excision) or after (30-week excision) expansion.

(I) Insulin tolerance was significantly worse after brown adipose tissue (BAT) ablation; n = 8 mice per excision cohort.

(J and K) Expression of (J) Irs1 and (K) Glut4 increases significantly in expanded brown adipose depot; n = 8 mice per cohort.

Equal numbers of males and females are represented. The boxplots display median, 1st and 3rd quartiles, and 1.5 x interquartile range for each cohort. Line graphs were generated using loess regression and smoothing. Individual data points are also represented. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S2.

(Lapa et al., 2017; Roberts-Toler et al., 2015; Shimizu and Walsh, 2015; Shimizu et al., 2014), we confirmed that the tissue expansion in SM/J mice has the expected properties of brown fat. Histological analysis of the fat depot taken from high-fat-fed SM/J mice at 30 weeks of age confirms the adipocytes in this expansion are brown adipocytes, with small multilocular lipid droplets and high UCP1 staining (Figures S2C–S2J). Expression of canonical brown adipose genes (Ucp1, Cidea, Eva1, Prdm16, Pgc1a) does not change between 20 and 30 weeks (Figures S2L–S2P), nor is there any evidence of a general “beiging” phenomenon in white adipose tissue (Wu et al., 2012). There is no significant difference in brown adipose tissue mitochondrial...
content (Figure S2K), core body temperature, or circulating free fatty acids between high- and low-fat-fed cohorts or between 20 and 30 weeks of age (Figures S2U and S2V). Additionally, although there are diet-dependent differences in the catecholamines norepinephrine and epinephrine, which activate UCP1-mediated leak respiration and non-shivering thermogenesis, there is no change in levels between ages in the high-fat-fed mice (Figures S2W and S2X). Thus, the interscapular adipose depot in high-fat-fed SM/J mice maintains a brown adipose identity after expansion that is not dependent on whole-animal beiging and is also not associated with altered thermogenesis.

Expanded Brown Adipose Tissue Serves as an Insulin-Stimulated Glucose Sink

If the brown adipose expansion is directly related to the glycemic resolution of the high-fat-fed SM/J mice, preventing or removing that expansion should revert their glucose parameters to an unhealthy state. To test these predictions, we removed the interscapular brown adipose depots from hyperglycemic 20-week-old and normoglycemic 30-week-old mice. Interestingly, removal of the brown adipose depot at 20 weeks does not affect serum glucose or insulin levels, or the glucose tolerance of the animals, indicating that the expanded brown adipose is downstream of the primary signal (Figures S2F–S2H) (Miranda et al., 2020). However, removal of the brown adipose depot before expansion prevents the natural improvement in insulin tolerance, and removal of the expanded tissue at 30 weeks results in a reversion to 20-week-old measurements (Figure 2I). These results strongly suggest that the expanding brown adipose tissue of obese SM/J mice serves as an insulin-stimulated glucose sink. This is supported by increased expression of both Irs1 and Glut4 (Figures 2J and 2K), key members of the insulin-stimulated glucose pathway (Pessin and Saltiel, 2000).

RNA Sequencing Reveals Enrichment of Differentially Expressed Cytokines and Genes Affecting Extracellular Matrix

Because the brown adipose tissue expansion is unique to high-fat-fed SM/J mice, we anticipated that there would be corresponding unique transcriptomic changes in the brown adipose. Indeed, we identified 267 genes whose expression significantly and uniquely changes between 20 and 30 weeks of age in high-fat-fed SM/J brown adipose tissue (at a 5% false discovery rate [FDR], out of 13,253 total genes expressed; Table S2). These expression changes occur when the mice resolve their glycemic dysfunction and expand their brown adipose depots. These genes are not differentially expressed in white adipose tissue taken from the same animals or in low-fat-fed SM/J controls (Figure 3A). Additionally, they are not differentially expressed in the LG/J strain of mouse, once again underscoring the genetic basis of the phenomenon (Table S3).

Over-representation analysis indicates these genes are enriched for those involved in cytokine-cytokine receptor interactions (p = 3.23e^{-6}), signaling receptor activity (p = 5.70e^{-6}), cell surface receptor signaling (p = 2.04e^{-7}), and extracellular matrix (ECM) components (p = 7.93e^{-13})(Figure 3B). Several genes belonging to these biological categories have evidence for their involvement in glucose homeostasis and change expression in a direction that is associated with improved metabolic health in high-fat-fed SM/J mice between 20 and 30 weeks of age (Figure 3C; Table S2). These are intriguing results because brown adipose has been identified as a source of cytokines that influence glucose homeostasis, and extracellular matrix changes are essential for tissue expansion, cellular signaling, and regulation of growth factor bioavailability (Lin and Farmer, 2016; Wang et al., 2015). However, extreme changes in ECM protein levels are associated with adipose dysfunction in obesity; thus, a fine balance between tissue remodeling and excessive accumulation of ECM proteins must be achieved to

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**Figure 3. High-Fat-Fed SM/J Mice Have Unique Brown Adipose Differential Expression between 20 and 30 Weeks of Age**

(A) Venn diagram illustrating the number of genes differentially expressed between high- and low-fat-fed 20- and 30-week-old SM/J interscapular brown or reproductive white adipose tissues.

(B) Terms enriched in genes differentially expressed by age in high-fat SM/J brown adipose colored by class: Gene Ontology Biological Process (GO_BP), Cellular Component (GO_CC), Molecular Function (GO_MF), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway.

(C) Heatmap of differentially expressed brown adipose tissue genes belonging to cytokine, extracellular matrix, or both gene ontologies. See also Tables S2 and S3.
maintain adipose tissue homeostasis (Sun et al., 2013). The direction of expression change in high-fat-fed SM/J mice reveals that their brown adipose expansion is associated with decreased expression of inflammatory (e.g., interleukin-7 receptor [II7R]) (Kim et al., 2014) and fibrotic markers (e.g., collagen type VIII alpha 1 chain [Col8a1], semaphorin 3C [Sema3c]) (Meijer et al., 2013; Sun et al., 2013), and changes in extracellular matrix components (e.g., matrix metalloproteinase 12 [Mmp12], procollagen c-endopeptidase enhancer [Pcolce]) (Huang et al., 2011; Lee et al., 2014) and cytokines (e.g., coagulation factor VII [F7], leptin [Lept], Sfrp1) (D’souza et al., 2017; Edén et al., 2015; Gauger et al., 2013) associated with metabolic health. Other mouse models of diet-induced obesity develop unhealthy brown adipose transcriptomes characterized by increased expression of pro-inflammatory genes and fibrotic markers (Alcalá et al., 2017). The direction of expression change in SM/J brown adipose tissue supports the uniqueness of this mouse model.

**Increased Sfrp1 Expression in Expanded Brown Adipose Tissue Correlates with Improved Glucose Tolerance**

We focused on Sfrp1 as a candidate gene that may underlie the increased brown adipose mass in SM/J high-fat-fed mice. Sfrp1 is a cytokine that inhibits Wnt/beta-catenin signaling. Wnt/b-catenin signaling promotes the fibroblast identity, and Sfrp1 blocks Wnt signaling to promote adipogenesis (Gauger et al., 2013; Lagathu et al., 2010; Wang et al., 2018). Its expression significantly increases in high-fat-fed SM/J brown adipose between 20 and 30 weeks, when the mice increase the size of their brown adipose depots and enter diabetic remission, and does not increase in white adipose of these same animals, or in the white or brown adipose of LG/J mice (Figures 4A and 4B; Figures S3A and S3B). Further, Sfrp1 expression significantly correlates with brown adipose depot mass and with improved glucose tolerance in high-fat-fed SM/J mice (Figures 4C and 4D). We performed a mediation analysis and found that the effects of Sfrp1 expression on glucose tolerance in high-fat-fed SM/J mice is significantly mediated by brown adipose mass (p = 0.004). Data from a human genome-wide association study (GWAS) revealed several variants in the SFRP1 locus, including one variant (rs973441) that is significantly associated with type 2 diabetes adjusted for body mass index (BMI) (Figure 4E) (Mahajan et al., 2018). There are several variants in the Sfrp1 locus between SM/J and LG/J genetic backgrounds, and the gene is located within the support intervals of QTLs associated with adiposity and glucose tolerance that were mapped in an F16 LG × SM intercross (Figure 4F) (Lawson et al., 2010, 2011). These data further underscore the role of SM/J genetic background has unique variants that contribute to its brown adipose expansion and diabetic remission. We hypothesize that DNA variants in SM/J mice increase Sfrp1, which in turn promotes brown adipogenesis by inhibiting Wnt/beta-catenin signaling, and this in turn may contribute to the tissue’s role as a glucose sink (Figure 4G).

**DISCUSSION**

Brown adipose has anti-diabetic properties that are conserved between mice and humans (Chechi et al., 2013; Cypess et al., 2009; Hanssen et al., 2016; van Marken Lichtenbelt et al., 2009; Saito, 2013; Saito et al., 2009; Stanford et al., 2013; Virtanen et al., 2009). Most brown adipose tissue research focuses on the effects of non-shivering thermogenesis on metabolic health. However, brown adipose coordinates a diverse array of physiological processes through the action of secreted factors associated with glucose homeostasis (e.g., fibroblast growth factor 21 [FGF21], bone morphogenic proteins [BMPs], Vascular endothelial growth factor A [VEGFA], interleukin 6 [IL-6], neuregulin 4 [Nrg4]) (Poekes et al., 2015; Villarroya et al., 2017, 2019; Kajimura et al., 2015). Further, the mechanism of insulin-stimulated glucose uptake into brown adipose tissue was well established before the discovery of beta-adrenergic stimulation of thermogenesis (Marette and Bukowiecki, 1993). This mechanism is independent of UCP1 activity (Inokuma et al., 2005; Olsen et al., 2019; Orava et al., 2011). Insulin-stimulated glucose uptake increases in animals with brown adipose transplants (Stanford et al., 2010) and is blunted under obese (Orava et al., 2013) and diabetic conditions (Lapa et al., 2017). Here we report a natural expansion of brown adipose tissue in obese mice that coincides not with increased thermogenesis, but with improved insulin sensitivity, likely through increased insulin-stimulated glucose uptake into the brown adipose tissue. We have shown corresponding increased expression of two critical members of the insulin signaling pathway: Glut4, which mediates insulin-stimulated, but not norepinephrine-stimulated, glucose uptake (Shimizu et al., 1996), and Irs1, which is also necessary for brown adipose differentiation (Fasshauer et al., 2001). It is possible that brown adipose expansion in high-fat-fed SM/J mice is a protective mechanism to improve insulin sensitivity before the hyperglycemia gets out of control.

We found that genes associated with cytokine activity are enriched in the expanded tissue and diabetic remission. Cytokine secretion likely contributes to the obesity-independent ability of SM/J’s brown adipose tissue to affect systemic glucose metabolism (Matsushita et al., 2014; Samms et al., 2015). We highlight Sfrp1 as a particularly intriguing cytokine that may underlie this phenomenon. Sfrp1 inhibits Wnt signaling, thus promoting adipogenesis (Ackers and Malgor, 2018; Bennett et al., 2002; Lagathu et al., 2010). Expression of Sfrp1 increases as adipocytes differentiate, proportional to the strength of adipogenic stimulation, and also increases during treatment with thiazolidinedione drugs, which generally act through improving insulin-mediated glucose metabolism (Lagathu et al., 2009). Although Sfrp1 has never been directly studied in brown adipose tissue, it is a member of a brown adipose coexpression module of genes enriched for cell division, with its expression strongly correlated with brown adipose mass in particular (Carson and Lawson, 2020).

In human obesity, SFRP1 expression is downregulated in subcutaneous white adipose tissue, correlating with impaired insulin sensitivity (Ehrlund et al., 2013). Further, its expression was found to be deficient in obese patients with glycemic dysfunction (Chen and Wang, 2018; Ehrlund et al., 2013; Lagathu et al., 2010). Finally, high-fat-fed Sfrp1−/− mice show dysregulation of glucose metabolism, including downregulation of Glut4 in white adipose tissue (Gauger et al., 2013). We show increased Sfrp1 expression in an expanding brown adipose depot that corresponds with increased Glut4 expression underlying potential
Figure 4. Increased Expression of Sfrp1 Is Likely Culprit of Brown Adipose Tissue Expansion

(A and B) Expression of Sfrp1 increases in (A) brown, but not (B) white, adipose tissue of high-fat-fed animals.

(C and D) Sfrp1 expression correlates with brown adipose mass across cohorts (C) and with improved glucose tolerance in high-fat-fed SM/J mice (D).

(E) Screenshot of variants in human adipose tissue in the SFRP1 locus region. Rs973441 is significantly linked with type 2 diabetes adjusted for BMI.

(F) The Sfrp1 locus region contains multiple variants between SM/J and LG/J strains and is located in the quantitative trait locus (QTL) associated with adipose weight and glucose tolerance mapped in an F16 intercross of the strains.

(G) Model of Sfrp1 acting to increase brown adipogenesis. Sfrp1 sequesters Wnt away from its receptor, leading to phosphorylation and degradation of beta-catenin and thus allowing an adipogenic transcriptional program. The boxplots display median, 1st and 3rd quartiles, and 1.5 x interquartile range for each cohort. Individual data points are also represented. Equal numbers of males and females are represented; n = 8–17 mice per cohort. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S3.
improvement in insulin-stimulated glucose uptake. Although previous studies of Sfrp1 have been conducted only in white adipose tissue, the data we present fit a model where brown adipocyte-secreted SFRP1 may act in an autocrine manner to stimulate brown adipose proliferation, leading to its role as an insulin-stimulated glucose sink and resulting in improved glucose tolerance and insulin sensitivity.

There is great interest in harnessing the potential of brown adipose to treat obesity and diabetes, either through the calorie-burning action of non-shivering thermogenesis or the endocrine action of adipokines. Research into the effects of brown adipose on systemic metabolism is in its infancy, and the community needs appropriate animal models to interrogate its physiological roles and identify potentially druggable targets. We present the SM/J mouse as a unique model to address this need. The SM/J mouse provides a tractable, genetic system in which to understand the relationship between brown adipose and glycemic control in obesity. Understanding this relationship in the SM/J mouse will open doors for identifying novel, potentially druggable targets for the improvement of glycemic control in humans.

**STAR★METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at [https://doi.org/10.1016/j.celrep.2020.108237](https://doi.org/10.1016/j.celrep.2020.108237).

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**AUTHOR CONTRIBUTIONS**

H.A.L. and C.C. designed the experiments. C.C., H.S., J.P.W., and H.A.L. performed physiological and molecular assays. C.C., M.A.M., and S.O. performed histological assays and analyses. C.C. and S.G. performed excision surgeries. C.C., J.F.M.-V., C.L.S.P., and H.A.L. analyzed the RNA-seq data. C.C. and H.A.L. wrote the manuscript. All authors edited and approved the final draft.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


STAR METHODS

KEY RESOURCES TABLE

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RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Heather A. Lawson, Ph.D. (email: lawson@wustl.edu).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
RNA-sequencing data generated for this study have been deposited at the Gene Expression Omnibus GSE157569 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157569).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

SM/J (RRID:IMSR_JAX:000687) and LG/J (RRID:IMSR_JAX:000675) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Experimental animals were generated at the Washington University School of Medicine and all experiments were approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Pups were weaned at 3 weeks and reared in same-sex cages of 3-5 animals until necropsy. At weaning, mice were randomly placed on a high fat diet (42% kcal from fat; Teklad TD88137) or an isocaloric low fat diet (15% kcal from fat; Research...
Diets D12284) (Table S1). Feeding was ad libitum. The animal facility operates on a 12 hour light/dark cycle with a constant ambient temperature of 21°C. All experiments were performed during the light phase. Mice of both sexes were used for all experiments. Age information for experimental animals can be found in each figure or table legend.

**METHOD DETAILS**

**Phenotyping**

Animals were weighed weekly until sacrifice. At 8, 18, 28, 38, and 48 weeks of age, animals were fasted for four hours (starting at 9am) before an intraperitoneal glucose tolerance test (starting at 1pm). At 19, 29, 39, and 49 weeks of age animals were subject to an intraperitoneal insulin tolerance test. At 10, 20, 30, 40, or 50 weeks of age, body composition was determined by MRI for SM/J mice. Body temperature of animals at 20 and 30 weeks of age was determined using a rectal thermometer. All animals were fasted for four hours, then given an overdose of sodium pentobarbital. Once the animals were anesthetized, blood was collected via cardiac puncture and euthanasia was achieved by cardiac perfusion with phosphate-buffered saline. After cardiac perfusion, tissues were collected and flash frozen in liquid nitrogen and stored at −80°C, or processed according to protocols for histology and other assays.

**Blood plasma assays**

Fasting blood glucose was measured using a GLUCOCARD Vital glucometer (Arkay, MN USA). ELISAs measuring plasma levels of free fatty acids (Wako Life Sciences 995-34693), leptin (Crystal Chem #90030), adiponectin (Crystal Chem #80569), IGFl (Crystal Chem #80574), and glucagon (Crystal Chem #81518) were quantified according to manufacturer’s protocol. Catecholamines were assayed through the Vanderbilt University Medical Center’s Hormone Assay and Analytical Services Core (https://www.vumc.org/hormone/assays; NIH grants DK059637 (MMPC) and DK020593 (DRTC)).

**Adipose histology**

At the time of tissue collection, small portions of interscapular brown and reproductive white adipose tissues were placed in 1 mL of neutral buffered formalin. These samples were incubated at 4°C while gently shaking for 24 hours. Immediately afterward, samples were placed into plastic cages and processed into paraffin blocks using a Leica tissue processor with the following protocol: 70% EtOH for 1 hour x 2, 85% EtOH for 1 hour, 95% EtOH for 1 hour x 2, 100% EtOH for 1 hour x 2, Xylenes for 1 hour x 2, paraffin wax. Adipose blocks were sectioned into 6 μm sections, with 2-4 slices on each slide.

**H&E Staining**

Slides were incubated at 60°C for 1 hour, then placed in xylene to remove remaining paraffin wax. Slides were then rehydrated using successive decreasing EtOH concentrations (xylene x 2, 100% EtOH x 2, 95% EtOH, 70% EtOH, H2O). Slides were incubated in hematoxylin (Leica Surgipath 3801570), Define (3803590), Blue Buffer 8 (3802915), and eosin (3801616), and dehydrated (95% EtOH, 100% EtOH, xylene x 2). Imaging was performed using the Zeiss AxioPlan2 microscope and Olympus DP software. Analysis of adipocyte size was performed using ImageJ. Images were converted to black and white and skeletonized to reveal only the cell wall outlines. Cell area was calculated from outlines with a lower limit of 50 um and upper limit of 700 um to reduce noise. All cells from a cohort (4-7 images each from 4 animals per cohort, equal numbers of males and females) were pooled for cell area density analysis.

**Immunofluorescence**

Slides were incubated at 60°C for 1 hour, then placed in xylene to remove remaining paraffin wax. Slides were then rehydrated using successive decreasing EtOH concentrations (xylene x 2, 50% EtOH in xylene, 100% EtOH x 2, 95% EtOH, 70% EtOH, 50% EtOH, 0.3% H2O2 in MeOH, H2O). Slides were washed with TBS and blocked in 10% normal donkey serum (Abcam ab7475) for 1 hour, followed by incubation with primary antibody overnight at 4°C. [Primary antibodies: rabbit anti-Ucp1 (1:100, Sigma U6382) and mouse anti-PHH3 (1:100, Invitrogen MA5-15220)]. After an additional wash, secondary antibody was applied for 1 hour at room temperature [Secondary antibodies: donkey anti-rabbit 488 (1:1000, Abcam ab150061) and donkey anti-mouse 647 (1:200, Abcam ab150107)]. Fluoroshield Mounting Medium with DAPI (Abcam) was applied to seal the coverslip and slides were stored at 4°C. Imaging was performed using the Zeiss Confocal microscope and Zen Lite imaging program. PHH3 analysis was performed using the CellProfiler program. Background was subtracted from DAPI and PHH3 channels using ImageJ. DAPI channel was used to identify total nuclei in CellProfiler. Adipose nuclei images were overlaid with PHH3 stain to identify mitotic adipose nuclei. Mitotic nuclei were summed across all 4 slides for each individual. Mitotic adipose index is reported as mitotic adipose nuclei divided by adipose nuclei multiplied by 100%.

**Quantitative rt-PCR**

Total RNA was extracted from brown, subcutaneous inguinal, and visceral reproductive adipose samples using the QIAGEN RNeasy Lipid Kit. High-Capacity cDNA Reverse Transcription Kit (Thermofisher) was used for reverse transcription. Quantitative-rPCR was performed to assess expression levels of target genes with an Applied Biosystems (USA) QuantStudio 6 Flex instrument using SYBR Green reagent. Results were normalized to L32 expression, which was experimentally determined to not be differentially expressed across diet and age cohorts. cDNA products were analyzed by the ∆Ct method. Primers: L32 forward TCCACAATGTCAGG
AGCTG, reverse GGGATTGGTACCTGATGG; Adipoq forward ACGTCATCTTCGCGGTGACT, reverse CTTAAGAGTAGCTGCA GTGACATCG; Cidea forward TGCTCTCTGTGATGGAATTG; reverse GCCGTGCTAAGAGATCGT; Eva1 forward
CCACTCTCTGTGATGATACACG; reverse GCATTGAGGACCTCTGCT; Fabp4 forward CACCTCTCTGATTGATG; reverse
AATGCTGATGCGCTTTGTGG; Pparg forward GAATGCGAGGTTCTCTCCAT, reverse TGCACCTCAGCAGCAGCATT; Prdm16 forward
CCACCAACAGGACTCTTACGACG; reverse GAGAGGACTCTGAGCTGCAA; Sfrp1 forward TACTGGCCCGAGATGCGAG; reverse
GAGGCTCTCGTGATATGG; Tbx1 forward GCCGAGGACAGAATGTTC, reverse TGTCATCTACGGGCCACAAAG; Ucp1 forward
CCTCCTCAAGTGGAATGTTGTA; reverse AGAGTTTAGAGGGAGGCCCC.

**Mitochondrial DNA quantification**

DNA was extracted from brown and inguinal adipose tissues using the QIAGEN DNeasy Blood and Tissue Kit. Briefly, 40mg of tissue was homogenized in 10% protease K through vortexing and incubation at 56 °C. DNA was precipitated with ethanol, collected in a spin column, and eluted in 150mL of buffer. DNA concentration was quantified on a Nanodrop, and 50ng was used in a qPCR reaction to quantify the amount of h19 (nuclear gene) and CytB (mitochondrial gene). Mitochondrial content was calculated as the ratio of mtDNA to nucDNA. Primers used: Cytb forward TCTACGCTAAATCCCAATAAC, reverse TTAGGCTCTGTTTGGAGGT; h19 forward TATGTGCCATCTGCTCG; reverse AAGGTTTAGAGGGGCG.

**RNA sequencing and analyses**

Sixty-four LG/J and SM/J mice were used for sequencing analysis, representing 4 males and 4 females from each diet (high and low fat) and age (20 and 30 weeks). Total RNA was isolated from interscapular brown and reproductive adipose tissues using the QIAshredder RNA spin columns, and eluted in 150mL of buffer. RNA concentration was measured via Nanodrop, and RNA quality/integrity was assessed with a BioAnalyzer (Agilent). RNAseq libraries were constructed using the RiboZero kit (Illumina) from total RNA samples with RIN scores > 7.5. Libraries were checked for quality and concentration using the DNA 1000LabChip assay (Agilent) and qualitative PCR, according to manufacturer’s protocol. Libraries were sequenced at 2x100 paired end reads on an Illumina HiSeq 4000. After sequencing, reads were de-multiplexed and assigned to individual samples.

FASTQ files were filtered to remove low quality reads and aligned against LG/J and SM/J custom genomes using STAR (Dobin et al., 2013; Nikolskiy et al., 2015). Briefly, LG/J and SM/J indels and SNVs were leveraged to construct strain-specific genomes using the GRC38.72-mm10 reference as a template. This was done by replacing reference bases with alternative LG/J and SM/J bases using custom python scripts. Ensembl R72 annotations were adjusted for indel-induced indexing differences for both genomes. Read counts were normalized via upper quartile normalization and a minimum normalized read depth of 10 was required. Alignment summaries are provided in Figure S4. Library complexity was assessed and differential expression between each age cohort for each strain–by-diet comparison was determined after TMM normalization in edgeR (Chen et al., 2014).

Functional enrichment of differentially expressed genes was tested by over-representation analysis in the WEB–based Gene Set Analysis Toolkit v2019 (Zhang et al., 2005). We performed analyses of gene ontologies (biological process, cellular component, molecular function), pathway (KEGG), and phenotype (Mammalian Phenotype Ontology). For each tissue, the list of all unique differentially expressed genes was tested against the background of all unique genes expressed in that tissue (Tables S2 and S3). A Benjamini–Hochberg FDR-corrected p value ≤ 0.05 was considered significant.

**Brown adipose excision**

Interscapular brown adipose tissue depots were removed from 20 or 30 week-old high fat-fed SM/J mice. A small longitudinal incision was made between the shoulder blades. All interscapular adipose tissue was carefully removed, and a cautering wand used to stop excessive bleeding when necessary. Surgeries were performed under general anesthesia by IP injection of ketamine/xylazine (100/200 mg/Kg) and mice were maintained in the surgical plane by isoflurane/oxygen for the duration of the procedure. Incisions were closed with 5-0 nonabsorbable sutures. Ketoprofen (2 mg/Kg) was provided post-procedure and topical antibiotic was applied to the incision daily for 3 days. Animal health and well-being was monitored daily. Sutures were removed at 10 days post-surgery. Mice were allowed to recover for 5 weeks after surgery or until they reached 30 weeks of age, then underwent a glucose tolerance test and an insulin tolerance test one week later. After an additional week of recovery, animals were sacrificed and plasma and multiple tissues harvested (reproductive and inguinal adipose depots, liver, heart, soleus, pancreas, hypothalamus) as described above.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data within individual cohorts were assessed for normality using a Wilks-Shapiro test. Outliers were identified by a Grubbs test (p < 0.05) and removed. The sex X diet X age term was not significant for any phenotype so males and females were pooled for analyses. For all box-plots, data were tested for significance among cohorts by ANOVA with a Tukey’s post hoc correction. A Welch’s unequal variances t test was performed between ages in each diet to determine significant differences in adipocyte cell size distribution. Mediation analysis was performed using the ‘mediation’ package in R. P values < 0.05 were considered significant. All statistical analyses were performed using the R software package.
Supplemental Information

Brown Adipose Expansion and Remission of Glycemic Dysfunction in Obese SM/J Mice

Caryn Carson, Juan F. Macias-Velasco, Subhadra Gunawardana, Mario A. Miranda, Sakura Oyama, Celine L. St. Pierre, Heather Schmidt, Jessica P. Wayhart, and Heather A. Lawson
Figure S1: Physiological characterization of SM/J and LG/J mice (Related to Figure 1).

Plasma levels of IGF1 (A), glucagon (B), adiponectin (C), and leptin (D) in high and low fat-fed SM/J mice at 20 and 30 weeks. Basal glucose (E) and glucose tolerance (F) of high and low fat-fed SM/J mice at 10, 20, 30, 40, and 50 weeks. Insulin tolerance (G) of high and low fat-fed SM/J mice at 20, 30, 40, and 50 weeks. Interscapular brown adipose depot weight in grams (H) and as a percentage of total body weight (I) of SM/J mice at 10, 20, 30, 40, and 50 weeks. Body weight (J) and brown adipose tissue as a proportion of total body weight (K) in high and low fat-fed LG/J mice at 20 and 30 weeks of age. Basal glucose (L) and glucose tolerance (M-N) of high fat-fed LG/J mice are higher than low fat-fed controls at 30 weeks. Insulin sensitivity (O-P) is not different among the LG/J cohorts. The boxplots display median, 1st and 3rd quartiles, and 1.5*Interquartile Range for each cohort. Individual data points are also represented. Number of animals per cohort = 8-16 for panels A-D, 21-73 for panels E-G, 16-25 for panels H-I, and 30-60 for panels J-P. Approximately equal numbers of males and females represented. Statistical differences calculated among all cohorts using one-way ANOVA with Tukey's post hoc correction. *p<0.05, **p<0.01, ***p<0.0001
Figure S2: Morphology and thermogenic characteristics of SM/J brown adipose tissue (Related to Figure 2)

Quantification of average cell size (A) and percentage of phosphohistone H3 positive cells (B) in brown adipose depots of high and low fat-fed SM/J mice. Representative images of brown and white adipose from high fat-fed mice at 20 (C-D) and 30 weeks of age (E-F) and from low fat-fed mice at 20 (G-H) and 30 weeks of age (I-J). Stained for H&E (left picture, scale bar = 200nm) or UCP1 (yellow) and DAPI (blue) (right picture, scale bar = 50nm). Mitochondrial copy number (K) was significantly higher in brown adipose tissue than in inguinal white adipose tissue at both 20 and 30 week time points with no significant difference between high or low fat-fed mice. Gene expression levels (L-T) quantified in three adipose depots of high fat-fed mice: intrascapular brown adipose (iBAT), inguinal white adipose (ingWAT), and reproductive white adipose (repWAT). Canonical brown adipose genes Ucp1 (L), Cidea (M), Eva1 (N), Pgc1a (O), and Prdm16 (P) show high expression in iBAT and no difference between 20 and 30 week-old mice. Beige adipose marker Tbx1 (Q) is not expressed in any depot. All adipose depots had roughly equal expression of general adipose markers Adipoq (R), Fabp4/AP2 (S), and Pparg (T). Body temperature (U) and plasma free fatty acid (V), norepinephrine (W) and epinephrine (X) concentrations at time of necropsy. The boxplots display median, 1st and 3rd quartiles, and 1.5*Interquartile Range for each cohort. Individual data points are also represented. Number of animals per cohort = 4 for panels A-B, 6-10 for panels K-T, and 8-21 for panels U-X. Approximately equal numbers of males and females represented. Statistical differences calculated using one-way ANOVA with Tukey’s post hoc correction among all cohorts in panels A-B and U-X and between 20 and 30 week cohorts within individual adipose depots for panels K-T. *p<0.05, **p<0.01, ***p<0.0001
Figure S3: Additional expression patterns of *Sfrp1* (Related to Figure 4).

Normalized expression for *Sfrp1* in brown (A) and white (B) adipose of LG/J mice. Correlation between brown adipose *Sfrp1* expression and mass in LG/J mice (C). n=8 animals per cohort. Equal numbers of males and females represented. The boxplots display median, 1st and 3rd quartiles, and 1.5*Interquartile Range for each cohort. Individual data points are also represented.
Figure S4: STAR alignment summaries for RNA-sequencing results (Related to STAR Methods)

Alignment summaries for RNA-sequencing results from (A) SM/J and (B) LG/J samples. Brown adipose samples are on the left, white adipose are on the right. Reads per million (left y-axis) are indicated by bars (yellow = uniquely mapped, green = multi loci, blue = mapped to too many loci) and % reads aligned (right x-axis) are indicated by red circles.
Table S1: High and low fat diet constituents (Related to STAR Methods). The diets (high fat: Teklad TD88137; low fat: Research Diets D12284) have been used in multiple studies of diet-induced obesity using the SM/J strain.

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<th>Source</th>
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<th>Low Fat*</th>
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<td>Energy from Fat</td>
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<td>Corn oil (g/kg)</td>
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<td>Anhydrous milkfat (g/kg)</td>
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<td>Cholesterol (g/kg)</td>
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<tr>
<td>Total energy (kJ/g)</td>
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# Teklad TD88137
* Research Diets D12284