Secreted frizzled-related protein 5 suppresses adipocyte mitochondrial metabolism through WNT inhibition

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Introduction

Obesity is a common disorder that predisposes individuals to type 2 diabetes, atherosclerosis, hypertension, and hyperlipidemia (1). The expansion of white adipose tissue (WAT) during obesity development is initially caused by an increase in adipocyte size, although with time, the total number of adipocytes increases due to preadipocyte differentiation (2, 3). It is well established that the inability to store excess energy in adipose tissue contributes to insulin resistance and metabolic complications (4, 5). Although regulation of adipocyte metabolism and differentiation have been extensively studied (reviewed in refs. 6–10), further understanding of molecular mechanisms influencing adipocyte biology is critical for our understanding and potential treatment of obesity and associated metabolic diseases.

Adipogenesis is the process by which mesenchymal precursor cells differentiate into adipocytes (11–13). It is well established that locally secreted and circulating factors regulate differentiation of precursors to adipocytes and also to alternative mesenchymal cell fates, such as osteoblasts (6). One of the most important regulators of adipogenesis is the WNT/β-catenin signaling pathway (14). Endogenous inhibitors of preadipocyte differentiation include WNT6, WNT10a, and WNT10b (15–18), which are expressed in precursor cells and decline during differentiation. In contrast, WNT5b and WNT4 are transiently induced during adipogenesis and act to promote this process (19, 20). Transgenic mice with expression of Wnt10b in adipose tissues are lean, are resistant to diet-induced and genetic obesity, have improved glucose homoeostasis and increased trabecular bone, and are resistant to osteoporosis (21–25). Conversely, Wnt10b−/− mice have a low bone mass phenotype (23) and transient expression of adipocyte markers during regeneration of skeletal myoblasts (26). Thus, numerous studies have demonstrated that WNT ligands are important regulators of mesenchymal cell fate, both in vitro and in vivo (27, 28).

The activity of WNTs is highly regulated by negative extracellular regulators, such as dickkopfs, WNT inhibitory factor 1, and secreted frizzled-related proteins (SFRPs) (27, 29). Of these, SFRPs have been the most extensively studied in the context of adipose tissue (16, 30–32). SFRPs have an N-terminal cysteine-rich domain that is homologous to frizzled proteins, the cell surface receptors for WNT ligands; these proteins also have a C-terminal netrin domain of unknown function (29). The SFRP family consists of 3 members in both human and mouse genomes, with SFRP1, SFRP2, and SFRP5 forming a subfamily based on sequence similarity within the cysteine-rich domain (33). SFRPs are thought to prevent downstream WNT signaling by binding to and sequestering WNT ligands in the extracellular space; however, under some circumstances, SFRPs may stimulate WNT signaling (33).

Sfrp1 expression increases during adipogenesis, is transiently stimulated by a high-fat diet (HFD), and is elevated with mild —
but not morbid — obesity (30). In 3T3-L1 preadipocytes, purified recombinant SFRP1 (and SFRP2) or ectopic SFRP1 expression inhibits WNT/β-catenin signaling and stimulates adipogenesis (16, 30). Sfrp5 is also induced during 3T3-L1 adipogenesis and is expressed more highly in isolated adipocytes than in stromal-vascular cells (31, 32, 34). Although 3 groups have reported that Sfrp5 is highly induced with genetic and/or diet-induced obesity (31, 32, 35), another found suppression of Sfrp5 under these conditions (34). Expression of Sfrp5 in WAT was also identified as one of the best a priori predictors of whether genetically identical C57BL/6J mice will gain adiposity when exposed to HFD (32). Sfrp5 mRNA expression is induced during adipogenesis and further increased with obesity. (A) RNA was isolated from EMSCs during adipogenesis (average Ct at day 12, 27.5) and with adipogenesis of ear mesenchymal stem cells (EMSCs) (36) isolated from the outer ears of mice (Figure 1A). Consistent with Sfrp5 induction during preadipocyte differentiation, and in agreement with previous studies (31, 32, 34), expression of Sfrp5 mRNA was markedly higher in the adipocyte fraction than in the stromal-vascular fraction of WAT from lean mice (Figure 1B). Based on Ct values derived from quantitative real-time RT-PCR (qPCR), expression in this context was approximately 10 times higher than in cultured adipocyte models (data not shown). This suggests that Sfrp5 may be expressed relative to adipocyte size, because primary adipocytes are much larger than cultured adipocyte models (data not shown).

Results

Sfrp5 mRNA expression is induced during adipogenesis and further increased with obesity. To investigate the role of SFRP5 in WAT biology, we first evaluated Sfrp5 expression during adipogenesis. We found that Sfrp5 mRNA was induced with differentiation of 3T3-L1 preadipocytes (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI63604DS1) and with adipogenesis of ear mesenchymal stem cells (EMSCs) (36) isolated from the outer ears of mice (Figure 1A). Consistent with Sfrp5 induction during preadipocyte differentiation, and in agreement with previous studies (31, 32, 34), expression of Sfrp5 mRNA was markedly higher in the adipocyte fraction than in the stromal-vascular fraction of WAT from lean mice (Figure 1B).

Figure 1

Sfrp5 mRNA is induced during adipogenesis and further increased with obesity. (A) RNA was isolated from EMSCs during adipogenesis (average Ct at day 12, 27.5). Values were normalized to Tbp mRNA, n = 7–11. (B) Sfrp5 mRNA was higher in adipocytes (AD; average Ct, 24.8) than the stromal vascular fraction (SV; average Ct, 33.0). n = 10. (C) Increased Sfrp5 mRNA in eWAT of 12-week-old male Lepy/db/db mice (Ct, 24.0) relative to Lepy/+ controls (Ct, 27.5). n = 7 per group. (D) Increased Sfrp5 mRNA in WAT after ovariectomy (Ovx). Shown is percent body fat 8 weeks after surgery and relative Sfrp5 mRNA expression in dorsolobar WAT of ovariectomized (Ovx). (E) Beginning at 8 weeks of age, mice were fed NCD or HFD for 12 weeks, and Sfrp5 mRNA was assessed in eWAT (NCD Ct, 30.2; HFD Ct, 19.91), peripheral WAT (pWAT), dorsolobar WAT (dWAT), brown adipose tissue (BAT), muscle, liver, pancreas, and kidney. n = 7 per group. (F) Assessment of Sfrp5 and Sfrp2 mRNA in eWAT from mice fed low-fat diet or HFD for 6 months (n = 10 per group) showed a positive correlation between Sfrp5 mRNA expression and percent body fat (R² = 0.84). (G) Positive correlation between adipocyte size and Sfrp5 mRNA (R² = 0.78). Leptin (R² = 0.5141) and Sfrp2 (R² = 0.1917) are shown for comparison. For A–E, values are mean ± SEM. *P < 0.05; **P < 0.01.
body fat percentage (Figure 1F, Supplemental Figure 1D, and ref. 32) and adipocyte size (Figure 1G). No relationship to adiposity was observed with Sfrp2, which was expressed in preadipocytes and declined during adipogenesis (Figure 1F and Supplemental Figure 1A). Taken together, these data provide compelling evidence that Sfrp5 mRNA is induced during adipogenesis, and that expression closely follows adipocyte growth and hypertrophy, both throughout development and with obesity.

Characterization of Sfrp5Q27stop mutant mice. To evaluate the role of Sfrp5 in adipose biology and obesity, we obtained Sfrp5Q27stop mice generated by N-ethyl-N-nitrosourea mutagenesis (38, 39). In these mice, a single base pair mutation results in a premature stop codon at glutamine 27 that is predicted to result in a nonfunctional allele (Supplemental Figure 2A). To test this prediction, we used immunoblotting to analyze Sfrp5 expression in WAT of control or Sfrp5Q27stop mice. In control mice, SFRP5 was detectable only after 20 weeks of HFD feeding (Figure 2A), consistent with the elevated Sfrp5 transcript levels observed under these conditions (Figure 1A). In contrast, SFRP5 was not detectable in HFD-fed Sfrp5Q27stop mice using antisera directed to either the N terminus (Figure 2A) or an internal antigen (data not shown). In addition, expression of Sfrp5 mRNA was decreased in adipose tissues and the adipocyte fraction of mutant mice (Figure 2B and Supplemental Figure 2B), which suggests that expression of Sfrp5 mRNA is maintained through a positive feedback mechanism. The well-known function of SFRPs as WNT inhibitors (29) suggests that Sfrp5Q27stop mice may have elevated WNT signaling, which might contribute to suppression of Sfrp5 mRNA. Consistent with this possibility, addition of recombinant WNT3a to cultured EMSC adipocytes rapidly suppressed Sfrp5 mRNA (Figure 2C). Finally, in light of the functional redundancy that exists for Sfrp1, Sfrp2, and Sfrp5 during development (40), we further evaluated expression of other Sfrp family members in Sfrp5Q27stop mice. This revealed a compensatory increase in Sfrp1, but not in Sfrp2, Sfrp3, or Sfrp4, in adipose tissue of mutant animals (Supplemental Figure 2C). Taken together, these data indicate that Sfrp5Q27stop mice have reduced expression of Sfrp5 mRNA and complete deficiency of SFRPS protein, with potential compensatory upregulation of Sfrp1.

SFRP5 is required for adipocyte hypertrophy, but not hyperplasia, during obesity. The phenotypes of control and Sfrp5Q27stop mice were indistinguishable when fed normal chow diet (NCD), as assessed by total body weight, tissue and organ weights, and body composition (data not shown); this may relate to the low levels of Sfrp5 mRNA and SFRP5 protein expression observed under this condition (Figure 1 and Figure 2A). Thus, we sought to investigate the role of SFRP5 by challenging Sfrp5Q27stop mice with HFD. Compared with controls, Sfrp5Q27stop mice resisted HFD-induced weight gain, with males demonstrating a stronger phenotype than females (Figure 2D). Notably, no change in body weight was observed in approximately 40% of cohorts (data not shown). Although some cohorts were statistically underpowered, we
Sfrp5 Q27stop significantly reduced in mental Figure 2D). However, the number of large adipocytes was within the adipose depot was similar between genotypes (Supplemental Figure 3B), but this metabolic phenotype was mild. We thus hypothesized that, as adipocytes reach their capacity to store lipid, they secrete SFRP5 to act in a paracrine manner to inhibit these WNTs and thereby recruit new adipocytes. To test this hypothesis, we performed histological analyses on eWAT in male Sfrp5 Q27stop mice. Consistent with the reduced adiposity we observed (Figure 2), serum leptin concentrations in male Sfrp5 Q27stop mice were significantly lower than in controls (Figure 3A). In addition, we monitored blood glucose concentrations in ad libitum–fed mice at 4-week intervals, starting with the first HFD feeding at 8 weeks of age. No significant differences in circulating glucose in either genotype were observed through 16 weeks of age (Figure 3B and Supplemental Figure 3A); however, by 20 weeks, glucose concentrations of male Sfrp5 Q27stop mice were lower than those of control mice (Figure 3B). In addition, male Sfrp5 Q27stop mice were resistant to HFD-induced hyperinsulinemia, a trend also observed in female Sfrp5 Q27stop mice (Figure 3C).

Ouchi et al. (34) suggest that Sfrp5–/– mice fed an obesogenic diet develop insulin resistance. To determine whether glucose homeostasis was altered in Sfrp5 Q27stop mice, we performed glucose tolerance and insulin sensitivity tests in mice fed HFD ad libitum (Figure 3D). Glucose tolerance in 20-week-old mice fed HFD. n = 14 (male); 14–15 (female). (E and F) Insulin tolerance test after a 3-hour fast. Mice were evaluated at (E) 20 (n = 14 [male]; 18–19 [female]) or (F) 48 (n = 8) weeks of age. For A–F, data are average ± SEM. *P < 0.05; **P < 0.01.

Loss of SFRP5 results in reduced leptin and mild improvements in glucose tolerance and insulin sensitivity. We next measured metabolic variables, such as circulating leptin, glucose, and insulin concentrations, to determine whether these were altered in HFD-fed Sfrp5 Q27stop mice. Consistent with the reduced adiposity we observed (Figure 2), serum leptin concentrations in male Sfrp5 Q27stop mice were significantly lower than in controls (Figure 3A). In addition, we monitored blood glucose concentrations in ad libitum–fed mice at 4-week intervals, starting with the first HFD feeding at 8 weeks of age. No significant differences in circulating glucose in either genotype were observed through 16 weeks of age (Figure 3B and Supplemental Figure 3A); however, by 20 weeks, glucose concentrations of male Sfrp5 Q27stop mice were lower than those of control mice (Figure 3B). In addition, male Sfrp5 Q27stop mice were resistant to HFD-induced hyperinsulinemia, a trend also observed in female Sfrp5 Q27stop mice (Figure 3C).

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To create a model in which we could investigate the potential relationship between SFRP5 and insulin sensitivity in vitro, we isolated EMSCs from control and Sfrp5Q27stop mice and differentiated them into adipocytes. As expected for a gene expressed late in adipocyte differentiation, loss of Sfrp5 did not influence adipogenesis, nor did it influence expression of common adipocyte markers such as PPARγ or FABP4 (Supplemental Figure 3, C and D). However, Sfrp5Q27stop adipocytes had reduced expression of Sfrp5 mRNA and a compensatory increase in Sfrp1 (Supplemental Figure 3E), consistent with our above-described observations in adipose tissue (Figure 2B and Supplemental Figure 2C). Under our cell culture conditions, loss of SFRP5 did not influence signaling events implicated in insulin resistance, including phosphorylation of Ser307-IRS1 or JNK (Supplemental Figure 3D), a signaling event implicated in insulin resistance, including phosphorylation of Ser307-IRS1 or JNK (Supplemental Figure 3D), a signaling event implicated in insulin resistance, including phosphorylation of Ser307-IRS1 or JNK (Supplemental Figure 3D), a signaling event implicated in insulin resistance, including phosphorylation of Ser307-IRS1 or JNK (Supplemental Figure 3D), a signaling event implicated in insulin resistance, including phosphorylation of Ser307-IRS1 or JNK. We therefore explored the potential role of SFRP5 in regulation of insulin sensitivity in vitro. We overexpressed Sfrp5 in 3T3-L1 adipocytes; however, we did not observe differences in phosphorylation of JNK or IRS1 with overexpression of SFRP5 (data not shown). Taken together, these data provided no support for the hypothesis that WNT5a or SFRP5 regulates insulin sensitivity of adipocytes.

**SFRP5 deficiency does not detectably alter energy balance.** We observed that SFRP5 was required for adipocyte growth under obesogenic conditions (Figure 2). To evaluate the effects of SFRP5 deficiency on whole-body energy balance, we measured metabolic rate in control and Sfrp5Q27stop mice using the Comprehensive Lab Animal Monitoring System (CLAMS). Despite a decrease in fat mass in Sfrp5Q27stop mice, differences in oxygen consumption and respiratory exchange ratio were not detectable between genotypes (Figure 4A and Supplemental Figure 4A). Differences in food intake were also not observed (Figure 4B). As SFRP5 deficiency did not cause detectable changes in whole-body energy balance, small, insensible alterations in food intake and/or energy expenditure over a period of many weeks likely cause the resistance to obesity observed in Sfrp5Q27stop mice.

**SFRP5 is a secreted protein tightly associated with the extracellular matrix.** SFRPs are generally believed to be secreted proteins that act through autocrine and paracrine mechanisms; however, the recent report by Ouchi et al. suggests that SFRP5 is detectable in culture media and serum, at least when overexpressed (34). Because adipose tissue appeared to be the major source of SFRP5 in obese mice (Figure 1E), it was important to establish whether SFRP5 influences energy metabolism in distal tissues and organs through an endocrine mechanism, similar to leptin or adiponectin, or whether its effects are exclusively local. To address these questions, we transiently transfected 293T cells with either empty vector or a vector containing a Sfrp5-Myc fusion construct; we also used the above-described 3T3-L1 cells, which stably overexpress Myc-tagged SFRP5 protein. SFRP5 was easily detected in whole-cell lysates and extracellular matrix fractions for 293T cells and 3T3-L1 adipocytes. In contrast, SFRP5 was detected in conditioned media of 293T cells only with long film exposures and very sensitive enhanced chemiluminescence, and could not be detected in 3T3-L1 adipocyte media even under these conditions (Supple-
Sfrp5Q27stop mice. To identify primary effects of SFRP5 ablation, rather than effects secondary to altered adiposity, we profiled mice with similar body masses between genotypes. Affymetrix microarray data were analyzed using Ingenuity Pathway Analyses to identify relevant biological networks or pathways (Supplemental Figure 5A). Gene profiling results indicated an upregulation of genes involved in mitochondrial oxidative phosphorylation (termed OXPHOS complex genes) in Sfrp5Q27stop mice, which raises the possibility that SFRP5 deficiency protects against obesity by increasing mitochondrial oxidative phosphorylation in adipocytes. qPCR analysis confirmed that RNAs encoding mitochondrial OXPHOS complex proteins, such as NADH dehydrogenase subunit 1 (Nadh1), NADH dehydrogenase subunit 5 (Nadh5), cytochrome c oxidase subunit 1 (Cox1), and ATP synthase FO subunit 6 (Atp6), were increased in the Sfrp5Q27stop samples evaluated by gene profiling (Supplemental Figure 5B) as well as in eWAT from an independent cohort of HFD-fed mice (Figure 5A). These effects of SFRP5 deficiency were cell-autonomous and specific to adipocytes, since we also observed elevated mitochondrial gene expression in cultured adipocytes (Figure 5B), but not in EMSC precursors (data not shown), from Sfrp5Q27stop mice. To determine whether elevated expression of these mitochondrial RNAs was due to increased number of mitochondria, we tested the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nucDNA) by qPCR in the cultured adipocytes. Although a significant increase in mitochondrial number was not always observed in Sfrp5Q27stop adipocytes, when all 6 independent experiments were taken together, the number of mitochondria increased approximately 20% (Figure 5C), consistent with the observed increase in mitochondrial gene expression (Figure 5B). These data suggest that Sfrp5Q27stop mice may restrain adipocyte growth under obesogenic environmental conditions due to increased number of mitochondria.

To evaluate the mechanism whereby SFRP5 deficiency increases mitochondrial copy number, we evaluated established mechanisms for increased biogenesis (51). Although differences in nuclear respiratory factor 1 (Nrf1), Nrf2, and ERRγ RNAs in cultured adipocytes derived from Sfrp5Q27stop mice were not observed (data not shown), the elevated expression of PPARY coactivator 1α (Pgc1α) (Figure 5D) was suggestive of a potential mechanism for increased mitochondrial biogenesis. This idea was supported by the increased expression of mitochondrial transcription factor A (Tfam) observed in both differentiated adipocytes and WAT from Sfrp5Q27stop mice (Figure 5, D and E), as Tfam is a well-established target of PGC1α that regulates both mitochondrial transcription and mtDNA copy number (52). Taken together, our results suggest that the increase in SFRP5 with obesity facilitates lipid storage by reducing mitochondrial biogenesis.

Elevated adipocyte respiration in SFRP5-deficient adipocytes is due to mitochondria number and functionality. Although white adipocytes...
have less oxidative capacity than do brown adipocytes, several reports have demonstrated the importance of white adipocyte mitochondria for adipogenesis and development of adiposity (53, 54). To further investigate whether loss of SFRP5 affects adipocyte oxidative capacity, we used a Seahorse XF Analyzer to measure oxygen consumption rate (OCR) of differentiated adipocytes and mitochondria isolated from cultured adipocytes or adipose tissue, both under basal conditions and in response to oligomycin, FCCP, and rotenone. Although basal OCR in Sfrp5 Q27stop EMSC adipocytes tended to be higher than in control adipocytes, this difference did not reach statistical significance (Figure 6A). However, both maximal OCR (after injection of the uncoupling agent FCCP) and respiratory capacity were significantly higher in Sfrp5 Q27stop than control adipocytes (Figure 6A), indicative of greater maximal aerobic capacity in Sfrp5 Q27stop than control adipocytes. These observations are consistent with SFRP5 deficiency causing increased mitochondrial number, but they could also result from increased mitochondrial functionality.

To determine whether increased respiratory capacity in EMSCs from Sfrp5 Q27stop mice is exclusively caused by increased mitochondrial number, we first measured OCR in isolated mitochondria from EMSC adipocytes. Interestingly, maximal consumption of oxygen in mitochondria from Sfrp5 Q27stop adipocytes was higher than that of controls (Figure 6B), despite similar numbers of mitochondria included in the assay for each genotype. These results were observed for functionality of both complex II (succinate plus rotenone) and complex I (malate plus pyruvate) (Figure 6B and Supplemental Figure 6B). We then extended this analysis to mitochondria isolated from eWAT. Mitochondria from HFD-fed Sfrp5 Q27stop mice had elevated OCR, with improved functionality for complex II and a trend for complex I (Figure 6C and Supplemental Figure 6B). These data suggest that increased respiratory capacity in Sfrp5 Q27stop EMSC adipocytes is due to increased mitochondrial biogenesis as well as improved aerobic capacity per mitochondrion.

WNT3a stimulates mitochondrial respiration and gene expression. Although SFRPs are well known to inhibit WNT signaling, they also influence differentiation and other cellular functions through a number of other mechanisms, such as inhibition of bone morphogenetic proteins (33, 55). To evaluate whether SFRP5 deficiency influences mitochondrial biology by increasing WNT signaling, we treated control EMSC adipocytes with recombinant WNT3a for 48 hours. We observed an increase in basal OCR with WNT3a treatment (Figure 7A). In addition, we evaluated a number of genes involved in mitochondrial biogenesis and function and found that WNT3a strongly induced expression of Nadh1, Nadh2, Cox1, and Atp6 (Figure 7B), as observed in Sfrp5 Q27stop EMSC adipocytes and adipose tissue (Figure 5). Next, we examined whether mitochondrial biogenesis markers are also regulated by WNT3a treatment. Similar to our results in Sfrp5 Q27stop EMSC adipocytes (Figure 5), WNT3a induced expression of both Pgc1α and Tfam (Figure 7C); however, unlike in Sfrp5 Q27stop EMSC adipocytes, WNT3a also elevated expression of Nrf1 and Nrf2. These experiments revealed...
some of the complexity in signaling and effects of WNT ligands in adipocytes and were consistent with the hypothesis that enhanced mitochondrial biogenesis and function in Sfrp5Q27stop mice was consistent with the hypothesis that enhanced some of the complexity in signaling and effects of WNT ligands in adipocytes and were consistent with the hypothesis that enhanced mitochondrial biogenesis and function in Sfrp5Q27stop mice.

### Discussion

The literature regarding the regulation of SFRP5 with obesity shows conflicting data. We found here that Sfrp5 mRNA was strongly induced in adipose tissues of 5 murine models of obesity, with increased SFRP5 protein levels also observed with diet-induced obesity (Figure 1 and Supplemental Figure 1). Our data are consistent with several prior publications (31, 32, 35) reporting elevated Sfrp5 mRNA with obesity. However, Ouchi et al. (31) were 5 and 16 weeks of age; thus, it is formally possible that the expression pattern changes dramatically between these ages and 20 weeks of age, as evaluated by Ouchi et al. (34). However, the growth chart from JAX labs for B6.V-Lepob/J mice (36) indicates obesity is well developed by 8 weeks, and only incremental increases in body weight occur after 16 weeks. Thus, the preponderance of evidence indicates that expression of Sfrp5 is induced during adipogenesis, is higher in isolated adipocytes than in the stromal-vascular fraction of WAT from lean animals, and increases dramatically with obesity development.

Although further study is required to establish the mechanistic basis for elevated SFRP5 in adipose tissue of obese mice, hints as to the mechanism come from our data that Sfrp5 mRNA expression is reduced in ZDF-Leprfa/fa mice (34) is intriguing in light of prior work indicating that SFRPs during early development of mice (40, 57). Moreover, the leukemia (58–62). However, the literature shows some of the complexity in signaling and effects of WNT ligands in adipocytes and were consistent with the hypothesis that enhanced mitochondrial biogenesis and function in Sfrp5Q27stop mice is caused by increased WNT signaling.

**Figure 7**

WNT3a stimulates mitochondrial respiration and gene expression. (A) Control EMSC adipocytes were treated with WNT3a (100 ng/ml) for 48 hours. Basal OCR and effects of oligomycin, FCCP, and rotenone are shown (representative of 5 independent experiments). (B and C) WNT3a induced mRNAs expression of (B) mitochondrial genes (Nadh5, Cox1, and Atp6) and (C) nuclear-encoded regulators (Pgc1a, Nrf1, Nrf2, and Tfam). Transcription expression was assessed by qPCR after exposure to WNT3a (100 ng/ml) for 48 hours. Values (normalized to Tbp mRNA) are expressed relative to control mice (n = 3 independent experiments). *P < 0.05; †P < 0.01.

Leprfa/fa rats and in Leprdb/db mice (34); although the literature shows no comparable data for SFRP5 expression in rat WAT, these Leprdb/db data are again discordant with ours and others’ (31). The Leprdb/db mice in our experiment were 12 weeks of age, and those of Lagathu et al. (31) were 5 and 16 weeks of age; thus, it is formally possible that the expression pattern changes dramatically between these ages and 20 weeks of age, as evaluated by Ouchi et al. (34). However, the growth chart from JAX labs for B6.V-Lepob/J mice (36) indicates that obesity is well developed by 8 weeks, and only incremental increases in body weight occur after 16 weeks. Thus, the preponderance of evidence indicates that expression of Sfrp5 is induced during adipogenesis, is higher in isolated adipocytes than in the stromal-vascular fraction of WAT from lean animals, and increases dramatically with obesity development.

Elegant work from the Kozak lab on variation in Sfrp5 gene expression in genetically identical C57BL/6J mice fed HFD suggests that expression of SFRP5 is regulated by epigenetic mechanisms (32). This idea is supported by the cancer literature, which indicates that methylation and inactivation of SFRP5 is associated with ovarian, gastric, breast, and renal cancers as well as myeloid leukemia (58–62). However, Sfrp5 upregulation is absent in 3T3-L1 adipocytes treated with a demethylating agent (35). Additionally, obesity does not affect methylation of specific CpG sites in the Sfrp5 promoter (35), although it is possible that regulation is from distant enhancers that remain to be evaluated.

The finding of Ouchi et al. that SFRP5 can be systemically delivered (34) is intriguing in light of prior work indicating that SFRPs act through autocrine and paracrine mechanisms (63). Our work
with overexpressed SFRP5 in 293T and 3T3-L1 cells demonstrated that, under these conditions, SFRP5 was easily detected in whole-cell lysates and extracellular matrix fractions and that significant detection in media was possible only when SFRP5 was competed off the extracellular matrix using heparin. These biochemical characteristics of SFRP5 are consistent with properties of other SFRPs (48–50). Compelling experiments in Xenopus indicate that SFRP5 readily diffuse within extracellular matrix of the developing embryo, which extends the gradient and signaling ranges of WNTs and BMPs (63). SFRP5 or other family members have not been detected in proteomic analysis of serum proteins (64, 65); however, a recent report suggests that circulating human SFRP5 can involve the β-catenin pathway, since β-catenin is suppressed during adipogenesis and targeted for ubiquitin-mediated turnover by PPARγ (15, 42). Furthermore, we did not observe increases in either IRS1 or MYC in WNT3a-treated adipocytes (data not shown). Instead, the mechanism in adipocytes appeared to be through induction of PGC1α, TFAM, and potentially other regulators of mitochondrial biogenesis and function, such as NRF1 and NRF2 (Figures 5 and 7), although these latter factors were not elevated in Sfrp5Q27stop mice. This mechanism is reminiscent of prior reports regarding the effects of WNT3a-conditioned media on osteogenesis (71), but contrary to our previous observations in brown adipocytes (72). Whereas PGC1α is well known for regulated induction of UCP1 (73), under no circumstances did we observe elevated UCP1 in our studies (data not shown). In addition to effects on mitochondrial biogenesis, increased maximum oxygen capacity was also observed in isolated mitochondria of Sfrp5Q27stop mice (Figure 6), which indicates that WNT signaling also causes functional changes to this organelle. In summary, inhibition of WNT signaling by SFRP5 was required to suppress oxidative metabolism and stimulate maximal adipocyte growth during obesity. Thus, SFRP5 deficiency in adipose tissue elevated WNT signaling to stimulate mitochondrial biogenesis and function, thereby resulting in mice resistant to adipocyte growth under obesogenic conditions.

Methods
Further information can be found in Supplemental Methods.

Animals and animal care. Sfrp5Q27stop mice were generated by N-ethyl-N-nitrosourea mutagenesis, in which a C79T mutation created a premature stop codon at glutamine 27 (38, 39), and the mutation was back-bred about 20 generations. Thus, only very closely linked secondary mutations would not have been eliminated. Sfrp5 was chosen for this functional study on the basis of its expression profile and close connection to adipocyte biology. Thus, the phenotype revealed was also expected; this would not be the case for a hypothetical second mutation, which would effectively have been chosen at random. To further address this possibility, we examined the 11 genes contained within the 400 kb surrounding Sfrp5; of these, only 3 showed a moderate level of expression in adipose tissue, based on publicly available tissue arrays. However, to our knowledge, there is no published literature on any of the genes related to adipose tissue. Thus, although a confounding second mutation cannot be excluded without sequencing the genome, it appears to be unlikely. Excision of the first exon of Sfrp5 to create the Sfrp5−/− line used by Ouchi et al. (34), as first described by Satoh (40), is not expected to directly interfere with expression of non-coding RNAs or other proteins, although it is conceivable that transcription factors binding to the exon could serve as enhancers for distal genes. An independent Sfrp5−/− mouse line has been created by Leaf et al. (57); however, these mice unfortunately no longer exist. Further studies using adipose-specific knockout of Sfrp5 will be required to shed light on the discrepancy between the phenotypes described herein for Sfrp5Q27stop mice and the Sfrp5−/− mouse line used by Ouchi et al. (34).

Mitochondrial biogenesis and remodeling is important for adipocyte differentiation and function (53, 54, 69), and to our knowledge, regulation of these processes by WNT signaling has not been previously reported in this context. We observed that WNT3a stimulated mitochondrial biogenesis and oxygen consumption in adipocytes. These effects appeared to be similar to those observed in C2C12 cells, in which WNT3a stimulates mitochondrial biogenesis through stabilization of β-catenin and induction of IRS1 and MYC (70). However, the mechanism in adipocytes is unlikely to involve the β-catenin pathway, since β-catenin is suppressed during adipogenesis and targeted for ubiquitin-mediated turnover by PPARγ (15, 42). Furthermore, we did not observe increases in either IRS1 or MYC in WNT3a-treated adipocytes (data not shown). Instead, the mechanism in adipocytes appeared to be through induction of PGC1α, TFAM, and potentially other regulators of mitochondrial biogenesis and function, such as NRF1 and NRF2 (Figures 5 and 7), although these latter factors were not elevated in Sfrp5Q27stop mice. This mechanism is reminiscent of prior reports regarding the effects of WNT3a-conditioned media on osteogenesis (71), but contrary to our previous observations in brown adipocytes (72). Whereas PGC1α is well known for regulated induction of UCP1 (73), under no circumstances did we observe elevated UCP1 in our studies (data not shown). In addition to effects on mitochondrial biogenesis, increased maximum oxygen capacity was also observed in isolated mitochondria of Sfrp5Q27stop mice (Figure 6), which indicates that WNT signaling also causes functional changes to this organelle. In summary, inhibition of WNT signaling by SFRP5 was required to suppress oxidative metabolism and stimulate maximal adipocyte growth during obesity. Thus, SFRP5 deficiency in adipose tissue elevated WNT signaling to stimulate mitochondrial biogenesis and function, thereby resulting in mice resistant to adipocyte growth under obesogenic conditions.

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Animals and animal care. Sfrp5Q27stop mice were generated by N-ethyl-N-nitrosourea mutagenesis, in which a C79T mutation created a premature stop codon at glutamine 27 (38, 39), and the mutation was back-bred about 20 generations onto the C57BL/6 background. C57BL/6, Lepob/ob and Lepob/ob mice were obtained from The Jackson Laboratory. Lepob/ob mice were as described previously (37). The following diets were used: NCD (catalog no. 5001, LabDiet; PMI Nutrition International); low-fat diet (10% calories from fat, catalog no. D12450B; Research Diets); HFD (45% calories from fat, catalog no. D12451; Research Diets Inc.). C57BL/6 mice were subjected to ovariectomy or sham operation at 3 months of age, as described previously (23). All mice were housed on a 12-hour light/12-hour dark cycle in the Unit for Laboratory Animal Medicine at the University of Michigan, with free access to water and diet.

Animal measurements. Blood glucose levels were determined using an automated blood glucose reader (Accu-Check; Roche). Serum insulin and leptin levels were measured by ELISA (Crystal Chem Inc.). Glucose tolerance tests were performed on mice that were fasted overnight (12 hours). Blood was collected immediately before as well as 15, 30, 60, and 120 minutes after i.p. injection of glucose (1 g/kg body weight). For insulin tolerance tests, mice were fasted for 3 hours and then injected with 0.5 U/kg body weight of human insulin (Novolin R; Novo Nordisk). Body fat, lean mass, and free fluid were measured in conscious animals using an NMR analyzer (Minispec LF90II; Bruker Optics). Oxygen consumption, carbon
dioxide production, spontaneous motor activity, and food intake were measured using CLAMS (Columbus Instruments International).

Analysis of adipocyte morphology. Ovarian adipose tissue from 20-week-old females was divided into 4 pieces (proximal side of ovary to distal), then fixed in 4% paraformaldehyde and paraffin embedded. For each piece, 3 independent sections spaced by 100 μm were stained with H&E, and 6–7 pictures were taken for each section. We counted more than 1,500 adipocytes per piece of WAT, resulting in a total of 6,700–8,900 adipocytes counted per mouse. Quantification of adipocyte size was done with ImageJ software (NIH). All images were converted into binary files using a unique threshold value that separated positively labeled cells from background.

Adipose tissue transplantation. Gonadal WAT (gWAT) was excised from control and Sfrp5Q27stop donor mice and transplanted subcutaneously into Lepr+/− recipient mice at 4 weeks of age. To control for host differences in hyperphagia, vasculature formation, or other variables, 100-mg pieces of tissue from 2 control and 2 Sfrp5Q27stop mice were transplanted into the same Lepr+/− recipient. After 10 weeks, the transplanted tissue was harvested and fixed in 4% paraformaldehyde. H&E-stained sections were subjected to morphometric analysis as described above.

Syntomal-vascular and adipocyte fractionation. Fractionation of WAT into stromal-vascular cells and adipocytes was performed as previously described (22, 74).

Cell culture. 3T3-L1 adipogenesis was as described previously (75). Cells that had been confluent for 2 days (assigned as day 0) were treated with 10% FBS with 0.5 mM methylisobutylxanthine, 1% tissue from 2 control and 2 hyperphagia, vasculature formation, or other variables, 100-mg pieces of tissue were collected from 20-week-old females was divided into 4 pieces (proximal side of ovary to distal), then fixed in 4% paraformaldehyde. H&E-stained sections were subjected to morphometric analysis as described above.

Immuno blot analysis. Tissue or cell extracts were immunoblotted with antibodies specific for SFRPS (SFRPS E19; Santa Cruz Biotechnology); laminin (Novus Biologicals); p-JNK, JNK, IRS1, p-S6 (Ser240/244), S6, p-AKT (Thr308), AKT, MYC, and ß-catenin (Cell Signaling); FABP4 (R&D Systems); PPARγ (Santa Cruz Biotechnology); and ß-tubulin (Sigma-Aldrich). p-IRS1 (Ser473) antibody was provided by L. Rui (University of Michigan). For SFRPS1 blots, we prepared concentrated adipose tissue lysates using Stratagene Clean Resin according to the manufacturer’s protocol (Agilent Technologies).

Plasmids. Sfrp5 was amplified from a mouse eye cDNA library using forward and reverse primers containing 5′ EcorI and 3′ Xhol restriction sites, respectively. For the Sfrp5-Myc fusion construct, the Myc tag sequence was inserted into the reverse primer so as to be in-frame at the C terminus of SFRPS5 protein. The resulting amplicons were cloned into the EcorI and Xhol sites of pcDNA3.1+ (Invitrogen) to generate pcDNA3.1+−Sfrp5-Myc constructs. For subsequent stable infection into 3T3-L1 preadipocytes, Sfrp5-Myc was subcloned into the pMSCVneo retrovector vector (Clontech Laboratories) using the EcorI and Xhol sites.

Reagents. Recombinant murine WNT3a, WNTs, and WNTsb were purchased from R&D Systems and each used at a concentration of 100 ng/ml. Oligomycin, FCCP, and rotenone were from Enzo Life Sciences. Adenosine diphosphate, succinate, malate, pyruvate, and antimycin A were purchased from Sigma-Aldrich.

mRNA quantification by RT-PCR. Total RNA was prepared from frozen tissue or cells by using RNA Stat60 according to the manufacturer’s protocol (Tel-Test Inc.). Genomic DNA was removed from total RNA using DNA-free Dnase 1 (Ambion Inc.). Total RNA was reverse transcribed with random hexamers (TaQMan Reverse Transcription kit; Applied Biosystems). qPCR was performed using the MqSS real-time PCR detection system with SYBR green reagents (Bio-Rad Laboratories). Primers for qPCR were as follows: Sfrp5 sense, 5′-GACCAACAATGTTGCTGTTG-3′; Sfrp5 antisense, 5′-GACCAACAATGTTGCTGTTG-3′; Sfrp1 antisense, 5′-ATCCCTCTGATGTCGAGACG-3′; Sfrp2 sense, 5′-ATGTTTGAGC-GTTCAACACACC-3′; Sfrp3 sense, 5′-ATGTTTGAGC-GTTCAACACACC-3′; Sfrp3 antisense, 5′-CGTTTCTCTCAATAAGATGTC-3′; Sfrp4 sense, 5′-AGAAGGTCCTACAAGTTGGAAG-3′; Sfrp4 antisense, 5′-GTTACTGCG-GACTGTTGGCGA-3′; Pgc1a sense, 5′-GTAGGGCCACGGTTGAGACGC-3′; Pgc1a antisense, 5′-GCTCTTTTGCGGTATTCATCCC-3′; Ndlb sense, 5′-CAGCTCCTTGACCCCCATATTCA-3′; Ndlb1 antisense, 5′-ATGCGTATGAGCAACAAT-3′; Ndlb5 sense, 5′-CTCTCAACAAAAGCACATCA-3′; Ndlb5 antisense, 5′-TTGAAGAATTGCAGGTCGATCA-3′; Cox1 sense, 5′-GAGAGGCTTTTGGTCTCTAAA-3′; Cox1 antisense, 5′-AGGTTTGTTCTCCGATGGT-3′; Ap6 sense, 5′-AGGTTTCCAATGTGTTGACGC-3′; Ap6 antisense, 5′-CTCTTGGTGTTGTGATTGACCA-3′. Other primers were described previously: Nfd2 and Tfam (78) as well as TATA box–binding protein (Tbp), hypoxanthine phosphoribosyltransferase 1 (Hprt), and 18S (76, 79).

qPCR to estimate mtDNA. DNA was prepared from frozen tissue or cells using Gentra Puregene Kits including RNase A treatment (Qiagen). mtDNA copy number per nuclear genome in EMSC adipocytes was quantified as described previously (80). PCR primers for amplification of nucleotides 314–489 of the mitochondrial genome were as follows: sense, 5′-AAGCGGCTTCATACGATTAAC-3′; antisense, 5′-CCCAAGTTGGGTGTTGTTTG-3′; Pgc1a sense, 5′-TTGAAGAATTGCAGGTCGATCA-3′; Pgc1a antisense, 5′-CTCTTGGTGTTGTGATTGACCA-3′. Other primers were described previously: Nfd2 and Tfam (78) as well as TATA box–binding protein (Tbp), hypoxanthine phosphoribosyltransferase 1 (Hprt), and 18S (76, 79).

Statistics. All data are presented as mean ± SEM and were analyzed by 2-tailed Student’s t test or ANOVA. Differences were considered significant for P values less than 0.05.
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