Expression of the Splicing Factor Gene *SFRS10* Is Reduced in Human Obesity and Contributes to Enhanced Lipogenesis

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SUMMARY

Alternative mRNA splicing provides transcript diversity and may contribute to human disease. We demonstrate that expression of several genes regulating RNA processing is decreased in both liver and skeletal muscle of obese humans. We evaluated a representative splicing factor, *SFRS10*, downregulated in both obese human liver and muscle and in high-fat-fed mice, and determined metabolic impact of reduced expression. *SFRS10*-specific siRNA induces lipogenesis and lipid accumulation in hepatocytes. Moreover, *Sfrs10* heterozygous mice have increased hepatic lipogenic gene expression, VLDL secretion, and plasma triglycerides. We demonstrate that *LPIN1,* a key regulator of lipid metabolism, is a splicing target of *SFRS10*; reduced *SFRS10* favors the lipogenic β isoform of *LPIN1*. Importantly, *LPIN1*β-specific siRNA abolished lipogenic effects of decreased *SFRS10* expression. Together, our results indicate that reduced expression of *SFRS10,* as observed in tissues from obese humans, alters *LPIN1* splicing, induces lipogenesis, and therefore contributes to metabolic phenotypes associated with obesity.

INTRODUCTION

Obesity is a global epidemic, with substantial adverse social, economic, and personal health consequences. Foremost among these are the severe metabolic complications of obesity, including insulin resistance, T2D (type 2 diabetes), and increased cardiovascular disease risk. While the precise molecular defects underlying these complications remain unknown, abnormal lipid metabolism is a consistent phenotype. Indeed, both hypertriglyceridemia and ectopic lipid accumulation in liver and skeletal muscle are closely linked to both insulin resistance and diabetes risk (Browning and Horton, 2004; Jacob et al., 1999; Kotronen et al., 2007). Thus, we aimed to identify expression signatures of obesity in liver and muscle of human subjects. We now demonstrate results from two independent human cohorts, which both identified decreased expression of genes regulating RNA processing and splicing as the top-ranking expression phenotypes in liver and muscle of obese humans.

RNA processing is a complex cascade including constitutive and alternative splicing, polyadenylation, and nuclear export of mature mRNA (Stamm et al., 2005). Alternative splicing occurs for more than 90% of human genes (Wang et al., 2008) in the spliceosome, which consists of pre-mRNA, small ribonucleoproteins (snRNPs), and two major groups of non-snRNPs, splicing factors, and heterogeneous ribonucleoproteins (HNRNPs). The resulting splice variants have a fundamental role in differentiation

and organ development (Bland et al., 2010), and tissue-specific isoforms are commonly observed (Blencowe, 2006; Nilsen and Graveley, 2010). Thus, alternative splicing should be viewed as an important adaptive mechanism used to create protein diversity in response to distinct developmental and metabolic cues (Salomonis et al., 2010; Nilsen and Graveley, 2010). Alternative splicing can be disrupted in several disease states, including cancer (Karni et al., 2007; Venables, 2004) and monogenic human diseases (Faustino and Cooper, 2003). Moreover, several genes linked to obesity and insulin resistance have been shown to be regulated by alternative splicing (Kishore and Stamm, 2006; Sesti et al., 1991; Lefai et al., 2001; Patel et al., 2005; Ghosh et al., 2007; Lee et al., 1996), and defects in RNA processing and nuclear export are associated with lipodystrophy (Agarwal and Garg, 2006).

Our results now indicate that modification of alternative splicing may also contribute to metabolic phenotypes associated with human obesity, as reduced hepatic and muscle expression of a subset of splicing factors is associated with obesity, increased hepatic fat content, and hyperinsulinemia. To investigate whether this downregulation could contribute to phenotypes associated with obesity, we further studied a representative splicing factor, SFRS10, which was downregulated in both liver and muscle of obese humans. *SFRS10* (official gene name, *TRA2B*), the homolog of *Drosophila* transformer-2 (*Tra2*), belongs to the SR-like protein family of splicing factors, and is an important modulator of alternative splicing of multiple genes (Nayler et al., 1998). We demonstrate that reduced expression of *SFRS10* alters splicing of *LPIN1*, a key regulator of lipid metabolism (Csaki and Reue, 2010; Péterfy et al., 2005; Yao-Borengasser et al., 2006; Ryu et al., 2009; Huang et al., 2011), and contributes to increased hepatic lipogenesis and increased VLDL secretion in mice.

RESULTS

Expression of Genes Regulating mRNA Processing Is Decreased in Obesity

To identify differentially expressed genes in both liver and muscle from insulin-resistant humans with obesity, we utilized high-density oligonucleotide arrays in two independent cohorts (Table 1). Up- or downregulated probesets common to both studies are shown in Table S1. GO-based pathway analysis (MAPPFinder) demonstrated that the top-ranking downregulated pathways in both tissues were related to RNA processing and splicing (Figure 1A). For example, 46 of 199 RNA splicing genes were downregulated in liver (Z score 7.5, adjusted $p < 0.001$) and 41 of 199 were downregulated in muscle (Z score 11.1, $p < 0.001$). Gene set enrichment analysis (GSEA) also identified the RNA-processing gene set as downregulated in obese subjects in liver (nominal $p < 0.001$, FDR 0.039), but not in muscle (nominal $p = 0.26$, FDR 0.87). Expression of 13 genes involved in RNA processing and splicing was decreased in both liver and muscle (Figure 1B). A complete list of genes for each tissue is shown in Tables S2 and S3.

We next asked whether decreased expression of RNA-processing genes is also present in a mouse model of diet-induced obesity. Indeed, expression of several RNA-processing genes was decreased following a high-fat diet (HFD) for 4 months in

Table 1. Clinical Characteristics of Study Subjects

	Liver Study		Skeletal Muscle Study	
	Lean NGT	Obese NGT/T2D	Lean NGT	Obese IGT/T2D
Men/Women	0/5	2/6	0/10	0/7
Age (years)	36 ± 12	43 ± 11	59.6 ± 5.0	60.0 ± 4.8
Body Mass Index (kg/m²)	24 ± 5	53 ± 7[b]	27.4 ± 5.4	31.8 ± 6.5[a]
Fasting glucose (mmol/L)	4.8 ± 0.8	6.5 ± 2.0	4.5 ± 0.6	5.0 ± 0.8
Fasting insulin (pmol/L)	36.8 ± 33.0	148.2 ± 132.6[a]	40.3 ± 20.2	73.0 ± 23.4[a]

Subjects had no known abnormality in glucose metabolism prior to study. Six obese subjects were diagnosed with type 2 diabetes (T2D) during the liver study. Four obese subjects were diagnosed with impaired glucose tolerance (IGT) and three with T2D during the muscle study. Data are mean ± SD.
[a] $p < 0.05$ versus lean of the same study.
[b] $p < 0.001$ versus lean of the same study.

both liver and muscle (Figure 1C). Reduced expression of SFRS10 and HNRPK was confirmed by western blot in liver of HFD-fed mice (Figure 1D).

Obesity in both humans and HFD-fed mice is characterized by alterations in both systemic and cellular glucose and lipid metabolism, which could potentially contribute to the observed downregulation of this subset of RNA-processing genes. We therefore assessed correlation of the 13 genes downregulated in both tissues with key metabolic phenotypes in humans (Table S4). Of these, liver expression of nine genes correlated inversely with obesity, as measured by body mass index (BMI), and five genes inversely with hepatic lipid content. Skeletal muscle expression of six genes correlated inversely with fasting insulin. No correlation between expression and fasting glucose was observed in either tissue.

We chose SFRS10 as a representative splicing factor for further analysis, as it was altered most consistently in both human and animal models. Examination of public databases indicated that hepatic mRNA expression of *Sfrs10* was not modulated in mice by (a) insulin deficiency (streptozotocin-induced diabetes) (Yechoor et al., 2002) or (b) experimentally induced insulin resistance (due to liver-specific deletion of insulin receptor IRS1, IRS2, or both IRS1 and IRS2) in the absence of obesity (Guo et al., 2009; Biddinger et al., 2008). Similarly, muscle-specific insulin resistance, even with superimposed hyperglycemia, does not alter *Sfrs10* expression (Yechoor et al., 2004). Additionally, exposure of HepG2 hepatoma cells and C2C12 myotubes to palmitate or elevated glucose did not affect *SFRS10* expression. However, overnight incubation with 10 nM insulin decreased *SFRS10* expression (Figure S1), supporting a potential role for chronic insulin exposure in contributing to decreased *SFRS10* expression.

Reduced Expression of *SFRS10* Leads to Increased Lipogenesis in Cultured Cells

To assess the functional consequences of decreased splicing factor gene expression, we experimentally reduced SFRS10
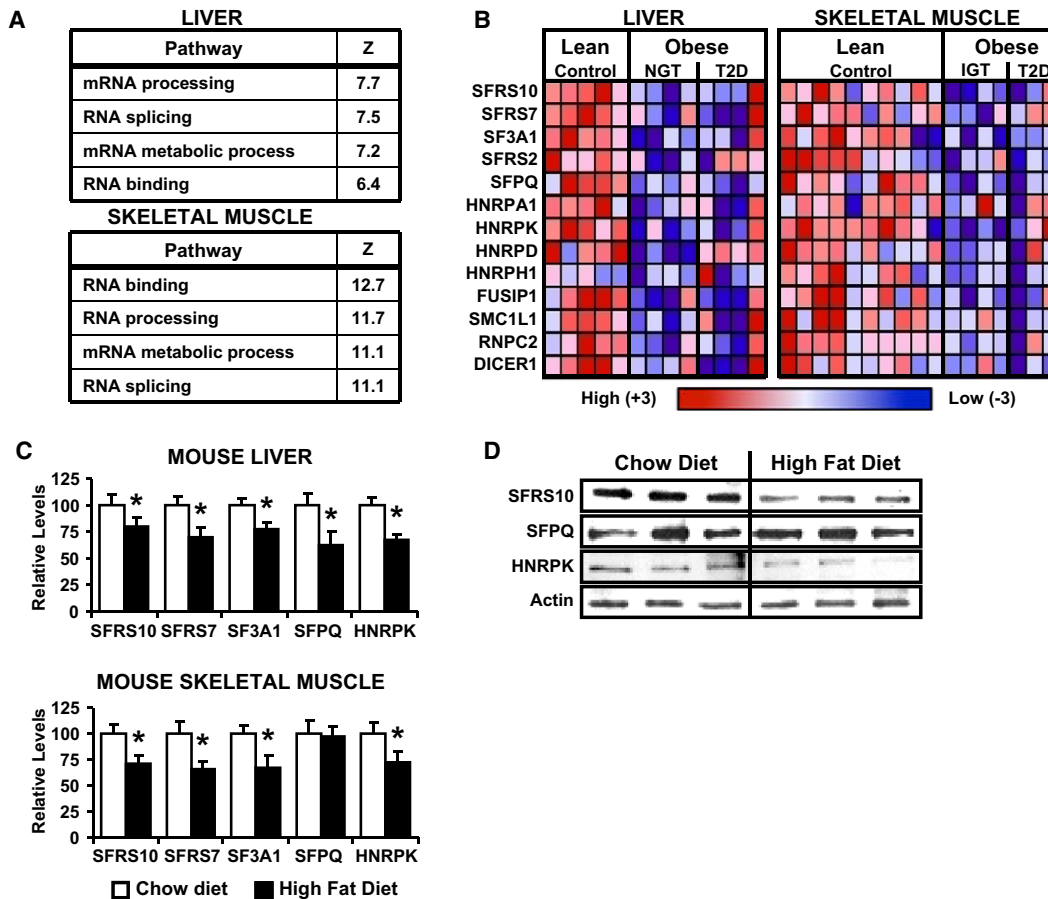
Figure 1. RNA-Processing Gene Expression Is Downregulated in Obesity

(A) Top-ranking downregulated pathways in obese humans are identified through GO-based pathway analysis (MAPPFinder) of microarray data from liver and muscle.

(B) Heatmap of 13 RNA-processing genes with decreased gene expression in both tissues. Blue indicates lower and red higher gene expression. NGT, normal glucose tolerance; IGT, impaired glucose tolerance; T2D, type 2 diabetes.

(C) Expression of RNA-processing genes was determined by real-time PCR from mouse liver and muscle after 4 months of HFD (black bars) compared to chow diet (white bars). Data are mean ± SEM. *, p < 0.05 versus chow (n = 6).

(D) Protein levels of SFRS10, SFPQ ,and HNRPK were measured by western blot from liver nuclear extracts. See Figure S1.

expression in HepG2 cells. siRNA-mediated knockdown of SFRS10 led to a 50%–70% decrease in mRNA and protein levels (Figure 2A). Given that decreased expression of several RNA processing genes was associated with increased hepatic lipid content in human subjects, we examined the effects of reducing SFRS10 on lipid metabolism in hepatic cells. Indeed, SFRS10 knockdown in HepG2 cells induced a 1.5- to 2-fold increase in lipogenic genes, including *SREBP1c*, *FASN*, *ACC1*, and *DGAT2* (Figure 2B). While we observed trends for SFRS10 knockdown to increase expression of *SREBP1a* and PCK1 (*PEPCK*), expression of other genes influencing lipid or glucose metabolism, including *ESR1* (*ERRα*), *NR1H3* (*LXRα*), *NR1H2* (*LXRβ*), *NR1H4* (*FXR*), *PPARA*, *PPARG*, *PPARD*, *PPARGC1A* (*PGC-1α*), and *PPARGC1B* (*PGC-1β*), did not differ following SFRS10 knockdown (Table S5). Notably, SFRS10 knockdown-mediated increases in lipogenic gene expression were accompanied by a 1.6-fold increase in lipogenesis, as measured by [14C]-acetate incorporation into the lipid fraction (p < 0.05) (Figure 2C), and led to a 1.4-fold increase in cellular

accumulation of triglycerides (TAG) (p < 0.05) (Figure 2D). TAG synthesis from [14C]-palmitate (Figure 2E) or fatty acid oxidation (Figure 2F) did not differ between control and SFRS10 knockdown, suggesting TAG accumulation was due to enhanced fatty acid synthesis. Similar effects of *SFRS10* knockdown were observed in C2C12 myotubes; these included induction of *SREBP1c* and *FASN* expression, as well as increased TAG accumulation (Figure S2). These lipogenic effects were specific for SFRS10, as knockdown of the constitutive splicing factor SF3A1 had no effect on lipogenic gene expression or lipid accumulation in either HepG2 or C2C12 cells (Figure S2).

To determine whether increased Sfrs10 expression could also modulate lipid metabolism, Hepa1c hepatoma cells were transfected with a DNA construct expressing *Sfrs10*. As seen in Figure S3, experimental overexpression of Sfrs10 in Hepa1c cells significantly decreased expression of the lipogenic genes *Fasn*, *Agpat2*, and *Dgat2* (p < 0.05 for SFRS10 versus GFP) (Figure S3B).
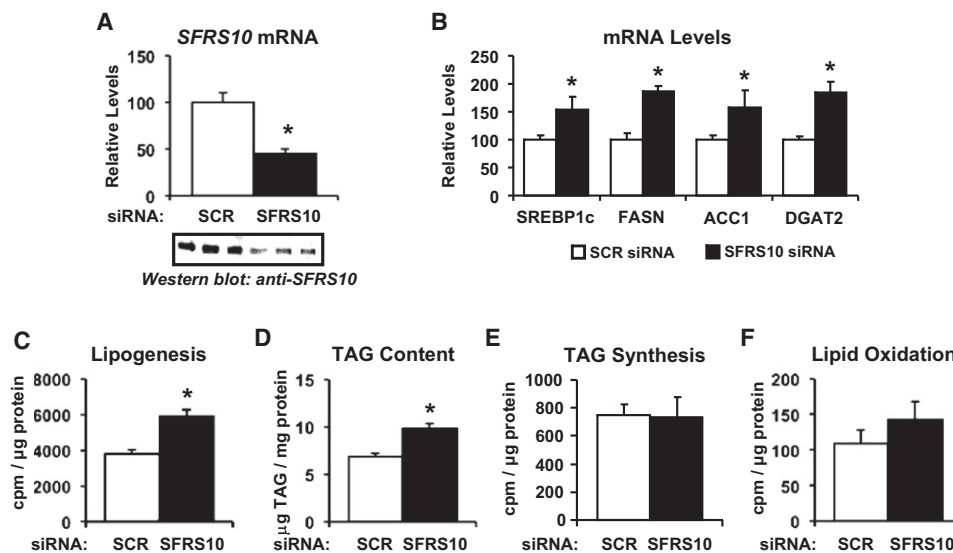
Figure 2. SFRS10 Knockdown Increases Expression of Lipogenic Genes and Leads to TAG Accumulation in Hepatic Cells
(A) (All panels) HepG2 cells were transfected with scramble (SCR) or *SFRS10* siRNA and analyzed 4 days later. SFRS10 mRNA and protein levels were analyzed by real-time PCR and Western blot (A).
(B) mRNA levels were determined by real-time PCR.
(C–F) Lipogenesis (from ^{14}C-acetate) (C), TAG levels (D), TAG synthesis (E), and fatty acid oxidation (F) were measured as described in Experimental Procedures. Data are mean ± SEM of triplicates, representative of three independent experiments. *, $p < 0.05$ versus SCR siRNA. See Figure S2.

Sfrs10$^{+/-}$ Mice Have Increased Lipogenic Gene Expression, VLDL Secretion, and Plasma Triglycerides

We next studied whether decreased expression of *Sfrs10* induces hepatic lipogenesis in a mouse model in vivo. For this purpose, the *Sfrs10* locus was disrupted as described in Experimental Procedures. No *Sfrs10*$^{-/-}$ pups were detected, confirming that genetic disruption of the *Sfrs10* gene is lethal during embryonic life (Mende et al., 2010). However, heterozygous *Sfrs10*$^{+/-}$ mice were viable, had normal growth and body weight, and showed no differences in blood glucose and insulin levels compared to wild-type animals (Figure S4). As shown in Figure 3A, hepatic *Sfrs10* mRNA levels were decreased by 30% in heterozygous mice compared to wild-type, a magnitude similar to the decrease in obese humans. Sfrs10 protein levels were only slightly decreased in the liver of heterozygous mice (Figure 3A), likely due to the known autoregulation of Sfrs10 protein mediated by a negative feedback loop (Stoilov et al., 2004). When present at high levels, Sfrs10 binds to its own exon 2, activating its inclusion and generating the *Tra2b4* isoform that is not translated into protein. Conversely, low Sfrs10 protein levels induce exon 2 skipping, favoring the *Tra2b1* isoform, the main functional isoform. In accord with this autoregulation, we observed an 80% decrease in *Tra2b4* mRNA levels in the liver of *Sfrs10* heterozygous mice, with a much smaller 25% decrease in *Tra2b1* (Figure S4). These data indicate that lower *Sfrs10* expression in the heterozygous mice promotes skipping of its own exon 2 in an attempt to restore protein levels.

Despite this autoregulation, and in agreement with the in vitro data, *Sfrs10* heterozygous mice showed increased hepatic expression of lipogenic and TAG synthesis genes, including *Srebp1c*, *Fasn*, *Scd1*, *Dgat2*, and *Agpat2* in the postprandial state (1.5- to 4.6-fold compared to wild-type, $p < 0.05$) (Figure 3B). While liver TAG accumulation remained unchanged (Figure 3C), plasma TAG levels were 52% higher in the heterozygous mice ($p < 0.05$) (Figure 3D). In particular, we observed a marked increase in plasma levels of the TAG-enriched VLDL fraction (3-fold, $p < 0.05$) (Figure 3E), indicating hepatic origin of the higher plasma TAG. To determine if this pattern was indeed due to increased hepatic VLDL secretion, we measured plasma TAG following administration of tyloxapol, an inhibitor of TAG clearance. Plasma TAG were 2- to 3-fold higher in heterozygous mice, confirming increased secretion ($p < 0.05$ at all time points) (Figure 3F). Together, these data indicate that decreased expression of *Sfrs10* is sufficient to increase hepatic lipogenic gene expression, increase VLDL secretion, and induce hypertriglyceridemia in a mouse model in vivo.

Lpin1 Splicing Is Regulated by Sfrs10

Our data suggest that specific targets of Sfrs10 upstream of *Srebp1c* and *Fasn* may mediate effects on lipid accumulation. It has been shown that Sfrs10 binds to RNA with the sequence NGAA. The protein binds to AGAA with an affinity of 2.25 μM and to GGAA with 4.5 μM (Cléry et al., 2011). Therefore, we considered known genes with alternatively spliced isoforms, particularly those regulating lipogenesis. Of these, *Lpin1* is known to regulate lipid metabolism (Csaki and Reue, 2010; Bou Khalil et al., 2010) and has two major alternatively spliced isoforms, α and β, and a third variant, γ, recently identified in brain (Han and Carman, 2010). The β isoform, generated by inclusion of exon 6, is associated with increased expression of lipogenic genes (Péterfy et al., 2005; Csaki and Reue, 2010). Interestingly, the alternatively spliced exon 6 of *Lpin1* contains a GGAA sequence motif that binds to SFRS10, and these sequences are highly conserved between human *LPIN1* and
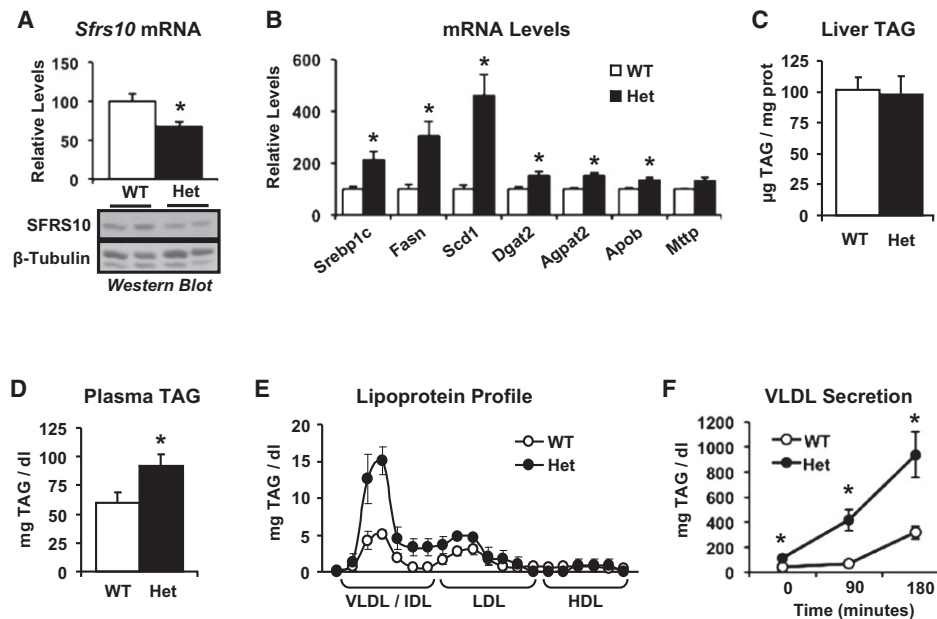
Figure 3. *Sfrs10* Heterozygous Mice Show Increased Lipogenic Gene Expression and Hypertriglyceridemia
(A) (All panels) Wild-type (WT) and *Sfrs10* heterozygous (Het) mice were fasted for 16 hr and then refed for 10 hr before sacrifice. Liver Sfrs10 mRNA and protein levels were determined by real-time PCR and western blot (A).
(B) Liver mRNA was quantified by real-time PCR.
(C and D) Liver TAG (C) and plasma TAG (D) were measured as in Experimental Procedures.
(E) Plasma lipoprotein profile was determined by FPLC.
(F) VLDL secretion was calculated by quantifying plasma TAG after Tyloxapol administration. Data are mean ± SEM of at least five mice/group and are representative of two independent cohorts. *, p < 0.05 versus WT. See Figure S4.

mouse *Lpin1* genes (Figure 4A). The location of the Sfrs10 site is unusual, as it partially overlaps with the U1 binding sites at the 5′ splice site. To determine if alterations in *SFRS10* expression could indeed modulate splicing of *LPIN1*, we used a minigene construct containing the alternative exon 6 of the human *LPIN1* gene. Cotransfection of the minigene construct with a plasmid expressing *SFRS10* caused exon 6 skipping (Figure 4B, left). Conversely, *SFRS10* siRNA increased inclusion of exon 6 (Figure 4B, right). These findings suggest that SFRS10 competes with U1 snRNA for binding at the 5′ splice site. With higher SFRS10 expression, U1 snRNA could be replaced by SFRS10, leading to exon skipping; with lower SFRS10 expression, U1 snRNA could bind the 5′ splice site and thus initiate exon inclusion.

We next assessed whether SFRS10 knockdown affected splicing of endogenous *LPIN1* in HepG2 cells. Notably, *SFRS10* siRNA did not change total *LPIN*1 expression (Figure S5), but altered the generation of *LPIN1* splice isoforms, yielding an increase in the β isoform in parallel with a modest decrease in the α isoform (Figure 4C, left). As a result, the ratio of the endogenous β isoform in relation to the α isoform (*LPIN1β/α* ratio) increased in response to SFRS10 knockdown (Figure 4C, right), confirming that modified SFRS10 expression leads to altered *LPIN1* splicing. A similar increase in *Lpin1β/α* ratio was observed in response to *Sfrs10* knockdown in C2C12 myotubes (Figure S2). This effect was specific to *SFRS10*, since knockdown of the constitutive splicing factor *SF3A1* did not alter *LPIN1* splicing, as indicated by unchanged

LPIN1β/α ratio (Figure S2). Similar results were observed in liver of *Sfrs10* heterozygous mice: no change in total *Lpin1* expression (Figure 4D, left), but an increase in the *Lpin1β/α* ratio (Figure 4D, right). Consistent with this effect, and in parallel with reduced SFRS10 levels, we also observed a significant increase in the *Lpin1β/α* ratio in liver of HFD-fed mice (Figure 4E) and a similar trend in liver of obese humans (Figure 4F). Conversely, overexpression of Sfrs10 in Hepa1c cells reduced the *Lpin1β/α* ratio as compared to GFP control (Figure 4G). Together, these results indicate that SFRS10 levels can modulate LPIN1 splicing and, thus, ratios between the β and α isoforms of LPIN1.

Lipin1 protein was initially identified as a type 1 phosphatidic acid phosphatase (PAP-1) acting in the TAG synthesis pathway (Han et al., 2006; Donkor et al., 2007), but also subsequently recognized as a transcriptional regulator of lipid metabolism (Finck et al., 2006). LPIN1β induces lipogenic gene expression in adipocytes (Péterfy et al., 2005). Indeed, Sfrs10 downregulation leads to increased expression of lipogenic genes in hepatoma cells (Figure 2B), C2C12 myotubes (Figure S2A), and in *Sfrs10* heterozygous mice (Figure 3B). However, we found no differences in hepatic PAP-1 activity (Figure S4) in heterozygous mice, as compared to wild-type mice, or in TAG synthesis from palmitate in cells (Figure 2E).

To investigate if the effects of *SFRS10* knockdown on lipogenic gene expression were mediated by increases in the *LPIN1β* isoform, we developed distinct siRNA oligonucleotides directed against either total *LPIN1* or specifically targeting exon 6, the *LPIN1β*-specific exon (Table S6). While total *LPIN1*
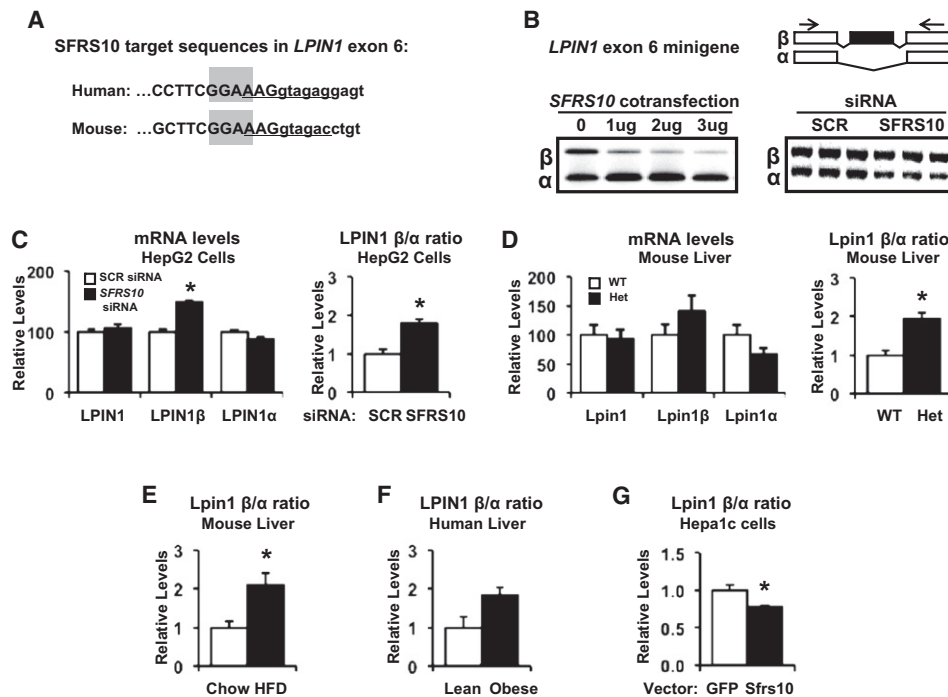
Figure 4. SFRS10 Regulates *LPIN1* Splicing

(A) The putative binding site of SFRS10, GGAA, is highlighted in gray within alternatively spliced exon 6 sequence (Ensembl release 61) of human and mouse *LPIN1*. The U1 snRNA binding site at the 5′ splice site is underlined.

(B) SFRS10 cotransfection increases exclusion of *LPIN1* exon 6 in a minigene system (left), while *SFRS10* siRNA increases inclusion (right). PCR primers are shown as arrows.

(C and D) Expression of total *LPIN1*, *LPIN1β*, and *LPIN1α* isoforms was determined (real-time PCR) in: HepG2 cells after SCR (white bars) or *SFRS10* siRNA (black bars) (C), and liver samples from WT (white, n = 7) and *Sfrs10* heterozygous (black, n = 5) mice (D).

(E–G) Expression of *Lpin1β* relative to *Lpin1α* was measured by real-time PCR in liver from: HFD (black, n = 6) and chow (white, n = 6) mice (E), lean (white, n = 6) or obese (black, n = 14) humans (F), and Hepa1c cells after GFP (white, n = 5) or SFRS10 (black, n = 5) overexpression (G). Data are mean ± SEM. *, p < 0.05 versus control. See Figure S2.

siRNA decreased both *LPIN1α* and *β* isoforms, the *LPIN1β*-targeted siRNA decreased *LPIN1β* expression by 68% without affecting *LPIN1α* expression, demonstrating specificity of the *LPIN1β* siRNA (Figures 5A and S5). Isolated knockdown of either total or the β-specific isoform of *LPIN1* had no significant effects on *FASN* expression. However, when *SFRS10* expression was also reduced (via cotransfection with *SFRS10* siRNA), the *LPIN1β*-specific siRNA abolished SFRS10-mediated increases in genes regulating fatty acid synthesis and TAG synthesis (Figure 5A). Importantly, *LPIN1β* knockdown also prevented the *SFRS10* siRNA-induced increase in lipogenesis (Figure 5B) and TAG accumulation (Figure 5C) and prevented increases in lysophosphatidic acid (LPA), an intermediate in the TAG synthesis pathway (Figure 5D). These findings indicate that effects of reduced SFRS10 on *LPIN1* splicing, favoring the *LPIN1β* isoform, are sufficient to increase expression of lipogenic genes and activate lipogenesis.

DISCUSSION

In the current study, we demonstrate downregulation of a subset of RNA processing genes in liver and skeletal muscle of obese humans. Expression of several splicing factors was inversely related to BMI, hepatic lipid accumulation, and hyperinsulinemia in humans. Moreover, diet-induced obesity reduced expression of several RNA-processing genes in mice, and exposure of cultured hepatoma cells to insulin reduced expression of SFRS10, suggesting a potential role for obesity-linked insulin resistance and/or chronic hyperinsulinemia in vivo. Additional factors, including genetic variation, could potentially contribute to obesity-associated differences in expression regulation. In particular, a polymorphism close to the *SFRS10* locus has been associated with obesity in several populations (Thorleifsson et al., 2009; Cheung et al., 2010).

To examine whether decreased expression of these splicing factors was merely a consequence of or could directly contribute to obesity-related metabolic phenotypes, we modulated expression of a representative alternative splicing factor, SFRS10, selected due to its consistent downregulation in both human and rodent tissues. Using both siRNA in cultured cells and in an in vivo mouse model of *Sfrs10* heterozygosity induced by gene targeting, we observed that experimental reduction in *Sfrs10* expression led to increased lipogenesis, likely mediated by upregulation of key lipogenic genes such as *Fasn* and *Srebp1c*. Moreover, we show that heterozygosity for *Sfrs10* increases VLDL secretion, resulting in marked hypertriglyceridemia in vivo—thus indicating that downregulation of SFRS10 can indeed contribute to disordered lipid metabolism.
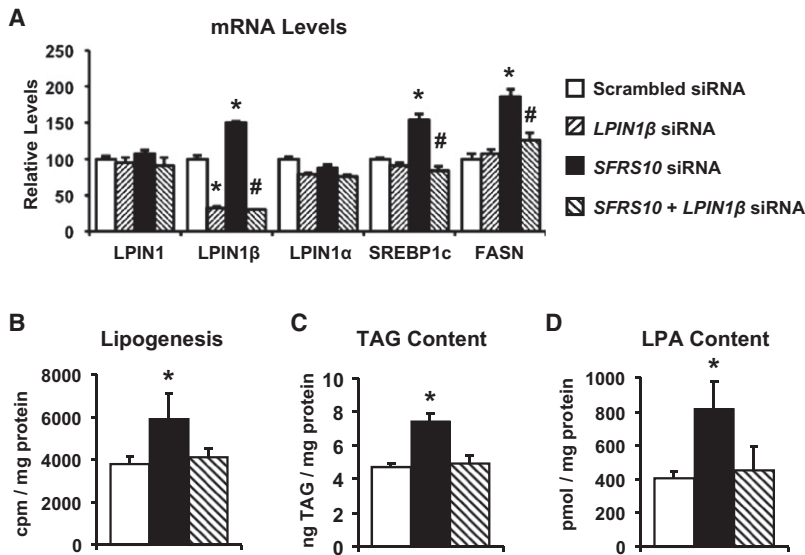
A

mRNA Levels

Figure 5. Increased Expression of Lipogenic Genes and Lipogenesis in Response to *SFRS10* siRNA Is Reversed with *LPIN1β* Knockdown

(A–D) HepG2 cells were transfected with the indicated siRNA and analyzed 4 days later. mRNA levels were determined by real-time PCR (A). Lipogenesis (B), TAG accumulation (C), and lysophosphatidic acid levels (D) were measured as in Experimental Procedures. Data are mean ± SEM of triplicates, representative of three independent experiments. *, $p < 0.05$ versus SCR siRNA. #, $p < 0.05$ versus SFRS10 siRNA. See Figure S5.

B
Lipogenesis

C
TAG Content

D
LPA Content

reduced *Sfrs10* expression in promoting lipogenesis are dependent upon the *Lpin1β* isoform. Thus, we propose a model in which reduced expression and splicing activity of Sfrs10 alters *Lpin1* splicing, favoring generation of the *Lpin1β* isoform and contributing to lipid synthesis and hypertriglyceridemia (Figure 6).

We have considered several possible mechanisms by which *Lpin1β*-dependent effects of reduced Sfrs10 expression could enhance lipogenesis and VLDL secretion. First, lipin1 has PAP-1 activity, yielding diacylglycerol (DAG) and thus promoting TAG synthesis (Donkor et al., 2007). However, we observed no change in TAG synthesis from labeled palmitate in HepG2 cells with Sfrs10 knockdown, and there were no differences in PAP-1 activity, nor in phosphatidic acid or DAG content (data not shown), in liver from Sfrs10 heterozygous versus wild-type mice. These data likely reflect the normal mRNA expression of Lpin1, 2, and 3 in the setting of reduced Sfrs10 expression. While we recognize that activity assays and static measures of PA and DAG do not assess potential increases in flux through the PA-DAG pathway, these data indicate that effects of Sfrs10 in promoting lipogenesis and hepatic VLDL secretion are unlikely to be mediated via increased lipin1-dependent PAP-1 activity.

A second mechanism potentially contributing to lipin1β-dependent effects on lipogenesis is its role as transcriptional coactivator. *Lpin1* overexpression in liver and adipose increases expression of genes modulating lipid oxidation (Finck et al., 2006; Donkor et al., 2008), potentially via interactions with PGC-1α and PPARα. In the context of *Sfrs10* reductions, *Lpin1β* effects on lipid oxidation are not observed. Rather, increased lipogenesis appears to be the dominant mechanism mediating TAG accumulation in HepG2 cells, as evidenced by *LPIN1β*-dependent increases in lipogenic gene expression and increased synthesis of TAG from acetate in response to SFRS10 knockdown. We did not observe increased TAG secretion into the medium in HepG2 cells (data not shown), possibly reflecting the impaired capacity of HepG2 cells to secrete TAG, compared to hepatocytes in vivo (Gibbons et al., 1994). However, the prominent effects of Sfrs10 on lipogenesis were confirmed in vivo, as demonstrated by the striking increases in lipogenic gene expression and parallel increases in TAG secretion and plasma VLDL-TAG in *Sfrs10* heterozygous mice. Our data support a role for differential transcriptional effects of the β versus α splice variants. While we do not yet fully understand mechanisms responsible for these effects, these splice variants
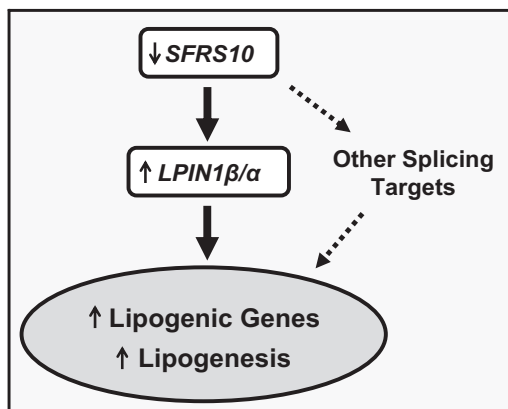
In this study, we identify *Lpin1* as a key splicing target of *Sfrs10*, which mediates its effects on lipogenesis. Originally, a *Lpin1* null mutation was discovered as a cause for lipodystrophy in mice (Péterfy et al., 2001); mutations in LPIN1 in humans have been linked to abnormal lipid accumulation in muscle (Zeharia et al., 2008). Subsequent studies identified *Lpin1* as both a phosphatidate phosphatase in the TAG synthesis pathway and also a transcriptional coactivator of PPARα (Finck et al., 2006; Csaki and Reue, 2010). Conversely, overexpression of *Lpin1* in either fat or muscle leads to obesity, with additional metabolic effects depending on tissue site of overexpression (Phan and Reue, 2005). In humans, transcriptional regulation of *Lpin1* alone is unlikely to explain hepatic lipid accumulation in obesity, as total *Lpin1* expression is reduced in parallel with insulin resistance in adipose tissue and liver (Croce et al., 2007; Donkor et al., 2008; Suviolahti et al., 2006; Yao-Borengasser et al., 2006) .

Alternatively spliced isoforms of *LPIN1*, including α and β, have distinct patterns of cellular localization and regulation (Péterfy et al., 2005; Bou Khalil et al., 2009; Han and Carman, 2010). For example, hepatic *LPIN1α* and β expression in humans does not correlate in individual subjects, indicating independent isoform regulation (Croce et al., 2007). The β isoform is associated with increased expression of lipogenic genes in adipose tissue (Péterfy et al., 2005) and has more prominent effects than the α isoform in promoting TAG secretion in hepatoma cells (Bou Khalil et al., 2009). By contrast, adenoviral expression of *Lpin1β* in primary hepatocytes modestly reduces TAG secretion (Chen et al., 2008). Furthermore, *Lpin1* isoforms have been shown to form homo- and hetero-oligomers (Liu et al., 2010). Thus, regulation of *Lpin1* expression, isoform splicing, and function is complex, and precise molecular mechanisms mediating these effects may be tissue-specific (Phan and Reue, 2005) and differ in fed/fasting transitions and between cellular and animal models. However, our studies using the *Lpin1* exon 6 minigene indicate that both knockdown and overexpression of *Sfrs10* alter *Lpin1* splicing, independently of *Lpin1* transcriptional regulation. Moreover, effects of

Figure 6. Human Obesity Is Associated with Decreased Expression of RNA-Processing Genes and Can Influence Metabolic Phenotypes
Reduced expression of the splicing factor SFRS10 alters splicing of *LPIN1*, leading to dysregulation of lipogenic pathways and contributing to hypertriglyceridemia. Other alterations in RNA processing in human obesity should be identified (dashed arrows).

have differential cellular localization (Péterfy et al., 2005); the Lpin1β polybasic motif may play a particularly important role in nuclear localization and regulation of gene expression, again *independently* of PAP-1 activity (Ren et al., 2010). Emerging data highlight the complexity of this system, as subcellular localization of Lipin1β in turn may be regulated by local phospholipase activity (Huang et al., 2011).

We recognize that general dysregulation of RNA-processing gene expression, as observed in our obese and insulin-resistant human cohorts, may have an impact on a broad range of cellular pathways and that we have focused solely on effects of *SFRS10* as a representative gene (Zhong et al., 2009). Furthermore, *LPIN1* is most likely just one of multiple potential splicing targets of SFRS10. In fact, numerous exons have the NGAA sequence that can serve as an SFRS10 binding site (Cléry et al., 2011). Identification of additional alternative splicing events mediated by SFRS10 and, more broadly, in human obesity, may provide targets that contribute to obesity or insulin resistance-associated phenotypes.

Additional mechanisms may also contribute to dysregulated expression and function of genes regulating mRNA splicing/processing in human obesity. For example, insulin may also regulate *SFRS10* expression, as overexpression of constitutively active Fox01 (resistant to nuclear exclusion by insulin) in liver increases expression of *Sfrs10* (Zhang et al., 2006). Furthermore, activation of Cdc2-like kinase family proteins (Clk) (Jiang et al., 2009) by insulin may alter phosphorylation and activity of splicing factors, including SFRS10 (Stoilov et al., 2004). Future studies are warranted to explore the potential interactions between insulin signaling, regulation of Clk kinases, and SFRS10.

In summary, we have now identified RNA-processing genes as a group of genes linked to human obesity. Using the *SFRS10-LPIN1* cascade as an example, we demonstrate that altered expression and splicing function of SFRS10 may modulate metabolic pathways critical for obesity and related metabolic phenotypes. These findings have several implications. First, genes and molecules regulating mRNA processing should be

investigated as potential candidates in obesity and insulin-resistant states. Second, alternatively spliced isoforms of known metabolic genes should be identified and characterized, as their alternative splicing may serve as an important regulatory step. A recent publication suggests sequence polymorphisms associated with obesity may also facilitate alternative splicing of obesity genes (Goren et al., 2008). Moreover, identification of pathways regulating alternative splicing in obesity may have implications for other chronic diseases linking with insulin resistance, as suggested for SFRS10 in Alzheimer's disease (Glatz et al., 2006). Finally, our study suggests that modulation of splicing factors and alternative splicing may be a potential therapeutic target for obesity-associated tissue lipid accumulation and consequent metabolic complications.

EXPERIMENTAL PROCEDURES

Human Subjects and Tissue Biopsies

Biopsies were obtained from two independent human cohorts: Boston (liver) and Finland (muscle). For the liver cohort, intraoperative biopsies were obtained from lean nondiabetic control subjects undergoing cholecystectomy (n = 5) and from obese subjects undergoing gastric bypass surgery (n = 8). Although these subjects had no known history of impaired glucose tolerance (IGT) or T2D, four of the obese subjects were diagnosed with T2D within 1 week prior to gastric bypass, on the basis of oral glucose tolerance testing (National Diabetes Data Group criteria [1979]). For LPIN1β/α ratio determination (Figure 4F), additional liver samples were obtained from subjects with similar metabolic profiles (Pihlajamaki et al., 2009). The muscle cohort consisted of 17 postmenopausal Caucasian women of Finnish ancestry who required cholecystectomy. Muscle biopsies (rectus abdominis) were obtained during abdominal surgery from lean postmenopausal women with normal glucose tolerance (NGT) (n = 10) or obese women who were diagnosed with IGT (n = 4) or T2D (n = 3) during the study. All subjects had normal liver, kidney, and thyroid function, no history of excessive alcohol intake, and no major chronic illness. All samples were washed with phosphate-buffered saline, immediately frozen in liquid nitrogen, and stored at $-80°C$.

Informed consent was obtained from all subjects after the purpose and potential risks of the study were explained. All human studies were performed in accord with the Helsinki Declaration and were approved by local institutional review, including by the Institutional Review Boards of the Joslin Diabetes Center and Beth Israel Deaconess Medical Center and the Ethics Committee of the Kuopio University Hospital.

Analytical Methods in Human Studies

Plasma glucose was measured using the glucose oxidase method (2300 STAT Plus, Yellow Springs Instrument Co. Inc., Yellow Springs, OH). Plasma insulin concentration was determined by radioimmunoassay (RIA, Diagnostic Systems Laboratories, Webster, TX) or commercial double-antibody solid-phase radioimmunoassay (Insulin RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden). Liver fat content was quantified from hematoxylin and eosin-stained sections (Pihlajamaki et al., 2009).

Animal Care and Treatment

All protocols were approved by the Joslin Institutional Animal Care and Use Committee. Mice were housed four per cage in an OLAW-certified animal facility, with 12 hr light cycles. For HFD experiments, 6-week-old ICR mice were placed on chow (17% calories from fat) or HFD (42% calories from milk-fat, Harlan Teklad) for 4 months.

Sfrs10 Mouse Generation and Analysis

Mice with heterozygous gene trap insertion at the *Sfrs10* locus (Tra2bGt(P142D08)Wrst) were generated using a mouse embryonic stem cell line (ID 3SP142D08, http://www.genetrap.org/cgi-bin/annotation.py?cellline=3SP142D08) containing a Rosabetageo vector inserted in intron 1 of the *Sfrs10* gene. Mice were generated by blastocyst injection and transferred to

pseudopregnant recipient dams. Sv129S2-derived offspring were inter-crossed with C57BL/6J mice. Genotyping was performed by PCR using tail DNA and two different primer pairs, one flanking the insertion site and the other recognizing the *neo* gene in the inserted sequence (available upon request). For plasma and tissue analysis, 8- to10-week-old males were fasted overnight and refed ad libitum for 10 hr. Blood glucose was measured using a Bayer Contour glucometer and insulin levels determined by ELISA (Crystal Chem Inc.). Mice were anesthetized with pentobarbital prior to tissue harvest.

Cell Culture

Human HepG2 cells, mouse Hepa1c cells, and mouse C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HepG2 and Hepa1c) or 20% fetal bovine serum (C2C12). To initiate differentiation, confluent C2C12 cells were incubated in DMEM containing 2% horse serum (Invitrogen). Full differentiation was observed by day five. For fatty acid incubations, palmitate (Alltech) was complexed with BSA, yielding a final stock of 5 mM.

RNA Isolation and Expression Analysis

Total RNA was isolated from human and mouse tissues with TRIzol (Invitrogen) and from HepG2 and C2C12 cells using RNeasy with DNase I treatment (QIAGEN). cDNA was synthesized and analyzed by real-time PCR (ABI Prism 7000 or 7700 Sequence Detection System, Applied Biosystems), using SYBR Green (cell/mouse) or TaqMan predesigned Assays-on-Demand (human). Primer sequences are available upon request.

For microarray analysis, 15 μg of cRNA were hybridized to human Affymetrix GeneChip HG-U133A arrays. Target preparation, hybridization, and scanning were performed in Joslin Diabetes Center Genomics Core (liver) or Turku Centre for Biotechnology (muscle). Signal intensities were quantitated using GeneChip Operating Software (GCOS). Global scaling was used to standardize signal intensities. MAPPFinder (www.genmapp.org) and GSEA (www.broad.mit.edu/gsea) were used to identify differentially expressed pathways and gene sets (Subramanian et al., 2005). For correlation analyses, Pearson correlations and associated p values for each probe set were calculated by comparison with permuted data (R version 2.0.1).

Protein Isolation and Western Blotting

Mouse liver or cultured cells were homogenized in buffer containing protease inhibitors (Sigma), sodium fluoride (100 mM), sodium orthovanadate (2 mM) and 1% Triton X-100. Protein concentrations were determined using BCA assay (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE for subsequent western blotting. Anti-SFRS10 antibody (S4070) was obtained from Sigma, and anti-SFPQ (ab11825) and anti-HNRPK antibodies (ab18195) from Abcam (Cambridge, MA).

siRNA of *SFRS10*, *SF3A1*, *LPIN1*, and *LPIN1β* Isoforms

HepG2 cells were transfected at 20%–40% confluency, and C2C12 myotubes were transfected on differentiation day three with 100 nM *SFRS10*, *SF3A1*, or *LPIN1* SMARTpool siRNA or siCONTROL nontargeting siRNA. DharmaFECT 3 (C2C12) or 4 (HepG2) transfection reagent was used (Dharmacon, Lafayette, CO). We designed three siRNAs specifically targeting exon 6 of *LPIN1* (Ensembl release 61, February 2011). The most specific and efficient siRNA targeting sequence, AAGAACUAGACAGACCUCCUU, was used for subsequent studies. To study siRNA knockdown effects on lipogenesis and lipid accumulation, cells were treated overnight with 1% BSA or 1% BSA/500 μM palmitate. Efficiency of transfection (>70%–80%; data not shown) was determined using siGLO RISC-Free nontargeting siRNA (Dharmacon).

DNA Constructs and Transfection

The mouse Sfrs10 cDNA (ATCC, Manassas, VA) was cloned into the pAdTrack-CMV vector, containing GFP as tracer. Hepa1c cells were transiently transfected using PolyFect (QIAGEN, ratio 3:1 DNA/Polyfect) with either pAdTrack-Sfrs10 or pAdTrack-CMV (control) plasmids. To enrich for Sfrs10-expressing cells, GFP-positive cells were selected through FACS (Flow Cytometry Core, Joslin) and mRNA analyzed from >100,000 GFP-positive cells.

In vivo Cellular Splicing Assay

The minigene was constructed using a fragment of human *LPIN1* exon 6, flanked by 500 nt intronic regions, which was cloned into an exon trap vector (Stoss et al., 1999). Effects of *SFRS10* on inclusion of *LPIN1* exon 6 were investigated by transfecting 1 ug of *LPIN1* exon 6 minigene into two cell models: (a) HepG2 cells (Mirus transfection reagent, Madison, WI) 2 days after *SFRS10* siRNA transfection (as above), and (b) HEK293 cells, together with *SFRS10*, using calcium phosphate. Cells were harvested the next day, and RNA was extracted (QIAGEN, Valencia, CA). cDNA was created using plasmid-specific primers, and PCR was done using minigene-specific primers flanking *LPIN1* exon 6.

Lipogenesis, TAG Synthesis and Accumulation, Palmitate Oxidation, PAP-1 Assay, Plasma Lipoprotein Profile, and VLDL Secretion

[^{14}C]-acetate (13 mM, 1 μCi/6-well plate, lipogenesis) or [^{14}C]-palmitate (50 nM, 5 μCi/6 well plate, TAG synthesis) was added to the media for 2 hr on day four after siRNA transfection. Cells were lysed as described above, and lipids were extracted with chloroform/methanol. Lipogenesis was assessed by measuring radioactivity in the lipid fraction. Thin-layer chromatography (TLC) with Silica Gel G plates (Analtech, Newark, DE) and hexane/diethyl ether/acetic acid (70/29/1) as a solvent was used to separate fractions. Incorporation of [^{14}C]-palmitate into the TAG fraction of conditioned medium was performed by lipid extraction and TLC separation of lipid classes (see above). To assess palmitate oxidation, cells were pretreated for 4 hr with 125 μM palmitate, followed by addition of [^{14}C]-palmitate (100 μM, 1 μCi/12-well plate); released CO_2 was trapped in 0.1 N KOH for 2 hr.

Mg^{+2}-dependent PAP-1 activity was assessed in Triton X-100 extracts from wild-type or SFRS10 heterozygous mice after an overnight fast and refeeding for 10 hr (Ren et al., 2010).

To measure TAG content from cells or liver tissue, lipids were extracted with chloroform/methanol, and total TAG content was determined using Triglyceride Assay Kit (Sigma). LPA levels were measured from flash-frozen cell pellets by mass spectrometry (Xiao et al., 2000). Plasma lipoprotein profile was determined by FPLC (Vanderbilt Diabetes Research and Training Center lipid core).

To measure VLDL secretion, mice were fasted overnight and refed for 2 hr. Mice were then fasted for 4 hr before intraperitoneal administration of Tyloxapol (Sigma) at a dose of 500 mg/kg. Tail vein blood samples were obtained before injection (time 0) and at 90 and 180 min for measurement of TAG (Sigma).

Statistical Analysis

Data analysis was performed with the SPSS/Win programs (version 10.0, SPSS Inc., Chicago, IL). A p value of <0.05 was considered statistically significant. Data are presented as mean ± SEM.

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