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

Jussi Pihlajamäki,1,4,13 Carles Lerin,1,7,13 Paula Itkonen,4 Tanner Boes,1 Thomas Floss,8 Joshua Schroeder,1 Farrell Dearie,1 Sarah Crunkhorn,1 Furkan Burak,1 Josep C. Jimenez-Chillaron,1 Tiina Kuulasmaa,4 Pekka Miettinen,6 Peter J. Park,2 Imad Nasser,3 Zhaiyi Zhang,10 Yan Xu,9 Wolfgang Wurst,8,11,12 Hongmei Ren,10 Andrew J. Morris,10 Stefan Stamm,10 Allison B. Goldfine,1 Markku Laakso,4 and Mary Elizabeth Patti1,*

1Research Division, Joslin Diabetes Center
2Children’s Hospital
3Beth Israel Deaconess Medical Center
Harvard Medical School, Boston, MA 02215, USA
4Department of Medicine
5Department of Clinical Nutrition
6Department of Surgery
University of Eastern Finland, Kuopio 70211, Finland
7Diabetes and Obesity Laboratory, IDIBAPS-CIBERDEM, 08036 Barcelona, Spain
8Helmholtz Zentrum München, Technische Universität München, Institut für Entwicklungsgenetik, Ingolstädter Landstrasse 1, 85764, Munich, Neuherberg, Germany
9Department of Obstetrics and Gynecology, Indiana University School of Medicine, Indianapolis, IN 46202, USA
10Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40536, USA
11MPI für Psychiatrie, Kraepelinstrasse 2-10, 80804 Munich, Germany
12Technical University Weihenstephan, Lehrstuhl für Entwicklungsgenetik, c/o Helmholtz Zentrum München and DZNE-site Munich, c/o Adolf Butenandt Institute, LMU, Schillerstrasse 44, 80336 Munich, Germany
13These authors contributed equally to this work
*Correspondence: mary.elizabeth.patti@joslin.harvard.edu
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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, LPIN1β-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; Kotroni 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 Gravelle, 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 Gravelle, 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 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
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), PPARα, PPARγ, PPARδ, PPARγC1α (PGC-1α), and PPARγC1β (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 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.
**Embryonic Life** (Mende et al., 2010). However, heterozygous
sfrs10 expression in the heterozygous mice promotes skipping of its
own exon 2 in an attempt to restore protein levels.

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).

**Sfrs10+/– Mice Have Increased Lipogenic Gene Expression, VLDL Secretion, and Plasma Triglycerides**

When present at high levels, Sfrs10 binds to its own exon 2, 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 M–1 and to GGAA with 4.5 M–1 (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

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**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 14C-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.
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 differences 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 LPIN1 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 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
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.
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 (Pétery 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 (Pétery 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 (Pétery 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 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, lpin1 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 lpin1-dependent PAP-1 activity.

A second mechanism potentially contributing to lpin1β-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 PPARγ. 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...
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 local-
ization of Lpin1β in turn may be regulated by local phospho-
lipase 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,
Lpin1β 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-associ-
ated phenotypes.

Additional mechanisms may also contribute to dysregulated
expression and function of genes regulating mRNA splicing/pro-
cessing 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 (Cik) (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 Cik 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,
genesis and molecules regulating mRNA processing should be
investigated as potential candidates in obesity and insulin-resis-
tant 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 associ-
ated 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 accumu-
lation and consequent metabolic complications.

**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
LPIN1β/α, leading to dysregulation of lipogenic pathways and contributing to hyper-
triglyceridemia. Other alterations in RNA processing in human obesity should
be identified (dashed arrows).

**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 determina-
tion (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
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 OЛАW-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
(Tra2b*3P142D08*tm1A0) were generated using a mouse embryonic stem cell
line (ID 3SP142D08, http://www.genetrap.org/cbg-bin/annotation.py?cellline=3SP142D08)
containing a Rosalabtagger vector inserted in intron 1 of the
Sfrs10 gene. Mice were generated by blastocyst injection and transferred to
pseudopregnant recipient dams. S129S2-derived offspring were intercrossed 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- to 10-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 (Altitech) 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 RNA were hybridized to human Affymetrix GeneChip HG-U133A arrays. Target preparation, hybridization, and scanning were performed in Joslin Diabetes Center Genomics Core (live) or Turkku 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 (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, LF3A1, LPIN1, and LPIN1β Isomorphism

HepG2 cells were transfected at 20%–40% confluence, and C2C12 myotubes were transfected on differentiation day three with 100 nM SFRS10, LF3A1, or LPINT 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 was determined by comparison with permuted data (R version 2.0.1).

### 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 μg 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

Incorporation of [14C]-acetate (13 mM, 1 μCi/6-well plate, lipogenesis) or [14C]-palmitate (50 mM, 5 μCi/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 assayed by measuring radioactivity in the lipid fraction. Thin-layer chromatography (TLC) with Silica Gel G plates (Anatech, Newark, DE) and hexane/ diethyl ether/acidic acid (70/29/1) as a solvent was used to separate fractions. Incorporation of [14C]-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 [14C]-palmitate (100 μM, 1 μCi/12-well plate); released CO2 was trapped in 0.1 N KOH for 2 hr.

### 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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