Inactivating hepatic follistatin alleviates hyperglycemia

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Unsuppressed hepatic glucose production (HGP) contributes substantially to glucose intolerance and diabetes, which can be modeled by the genetic inactivation of hepatic insulin receptor substrate 1 (Irs1) and Irs2 (LDKO mice). We previously showed that glucose intolerance in LDKO mice is resolved by hepatic inactivation of the transcription factor FoxO1 (that is, LTKO mice)—even though the liver remains insensitive to insulin. Here, we report that insulin sensitivity in the white adipose tissue of LDKO mice is also impaired but is restored in LTKO mice in conjunction with normal suppression of HGP by insulin. To establish the mechanism by which white adipose insulin signaling and HGP was regulated by hepatic FoxO1, we identified putative hepatokines—including excess follistatin (Fst)—that were dysregulated in LDKO mice but normalized in LTKO mice. Knockdown of hepatic Fst in the LDKO mouse liver restored glucose tolerance, white adipose tissue insulin signaling and the suppression of HGP by insulin; however, the expression of Fst in the liver of healthy LTKO mice had the opposite effect. Of potential clinical significance, knockdown of Fst also improved glucose tolerance in high-fat-fed obese mice, and the level of serum Fst was reduced in parallel with glycated hemoglobin in obese individuals with diabetes who underwent therapeutic gastric bypass surgery. We conclude that Fst is a pathological hepatokine that might be targeted for diabetes therapy during hepatic insulin resistance.

The complex pathophysiology of type 2 diabetes (T2D) arises from cell-autonomous consequences of insulin resistance that can propagate among heterologous tissues through dysregulated lipid flux, bile acids or circulating intermediary metabolites, as well as dysregulated production and secretion of cytokines, adipokines and hepatokines. Although T2D is characterized by systemic insulin resistance, disruption of hepatic insulin signaling alone recapitulates many aspects of T2D, including enhanced endogenous glucose production. Even though endogenous glucose production includes renal and intestinal contributions, it is mainly a function of hepatocytes. Hepatic glucose production (HGP) is suppressed directly by insulin via inhibition of hepatic glycogenolysis and gluconeogenesis, and indirectly by insulin-mediated suppression of adipose tissue lipolysis, glucagon secretion and neuronal signals.

The proteins insulin receptor substrate 1 (Irs1) and Irs2 link the activated insulin receptor kinase (IRK) to the phosphatidylinositol-3-OH kinase (PI3K)–Akt cascade, which regulates the expression of hundreds of hepatic genes by inactivating the Forkhead box O (FoxO) family of transcription factors. We previously investigated the consequences of hepatic Irs1 and Irs2 deletion in LDKO mice and the complementary LTKO mice. In addition to dysregulated hepatic metabolism and mitochondrial dysfunction, LDKO mice develop systemic metabolic disease—including glucose intolerance, hyperinsulinemia and dysregulated energy homeostasis. Remarkably, hepatic and systemic dysregulation is largely corrected upon further genetic deletion of hepatic Foxo1 in the LDKO mice—despite continued persistent and complete hepatic insulin resistance. These findings are confirmed and extended by others using compound hepatic-specific IRK; FoxO1-knockout or Akt1; Akt2; FoxO1-knockout mice. The normalization of HGP in these models owes, at least partially, to the re-sensitization of white adipose tissue (WAT) to insulin, which indirectly reduces hepatic gluconeogenesis by decreasing substrate availability and downregulating hepatic pyruvate carboxylase activity.

In models of complete hepatic insulin resistance (including LTKO mice), genetic disruption of hepatic Foxo1 substantially normalizes the expression of hundreds of dysregulated hepatic genes. Within such mice, FoxO1-dependent gene expression, or metabolic signals generated in hepatocytes, could reasonably contribute to dysregulation of peripheral insulin sensitivity and promote the delivery of metabolic intermediates to the liver—including excess glycerol and free fatty acids (FFAs) from adipose tissue. Thus, though it fails to restore hepatic insulin signaling per se, disrupting hepatic Foxo1 in LDKO mice (that is, LTKO mice) and similar models effectively restores glucose tolerance.

Regardless, how hepatic FoxO1 disruption might prevent the manifestation of peripheral insulin resistance has remained unclear.

Comprehensive analyses of gene expression reveal various genes encoding secreted proteins that are expressed in the human liver (so-called hepatokines), some of which might affect glucose and lipid metabolism. We hypothesized that FoxO1-dependent dysregulation of hepatokines in the LDKO liver might promote peripheral insulin resistance—and substantially underlie the restoration of metabolic homeostasis in LTKO mice. In this report, we use the LDKO and LTKO mice to identify follistatin (Fst)—best known for its modulation of transforming growth factor-β (TGF-β) superfamily members—as a key FoxO1-dependent hepatokine that can dysregulate WAT insulin sensitivity and hepatic gene expression to propagate systemic metabolic disease during hepatic insulin resistance.

**Results**

Hepatic Foxo1 is required for excess HGP and glucose intolerance during hepatic insulin resistance. Lipolysis of WAT

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triglycerides, which is suppressed by insulin, indirectly promotes hepatic glucoseoneogenesis. To establish whether WAT insulin resistance alone dysregulates systemic glucose tolerance, we produced FDKO mice by intercrossing Irs1<sup>L/L</sup>; Irs2<sup>L/L</sup> mice (Cntr) with adipose-specific Cre<sup>Adip</sup> mice (adiponectin promoter-driven Cre mice) to delete Irs1 and Irs2 specifically in adipocytes (Supplementary Fig. 1a). Compared to floxed Cntr, LDKO mice (Irs1<sup>L/L</sup>; Irs2<sup>L/L</sup>; Cre<sup>L/L</sup>; Cre<sup>Adip</sup>), the FDKO mice displayed normal glucose tolerance (Supplementary Fig. 1b), a result that is similar to mice lacking the adipocyte insulin receptor (FIRKO mice)<sup>5</sup> or adipocyte Akt1 and Akt2 (ref. 2). Thus, dysregulation of the Irs1–2–PI3K–Akt cascade in WAT alone was insufficient to impair systemic glucose homeostasis during the intraperitoneal glucose tolerance test (GTT).

To understand the requirement of hepatic insulin signaling for systemic glucose homeostasis, we investigated glucose tolerance and HGP in LDKO and LTKO mice. As shown previously, LDKO mice developed glucose intolerance, which was corrected in LTKO mice (Supplementary Fig. 1b,c). As excess HGP contributes to systemic glucose dysregulation, we used a hyperinsulinemic-euglycemic clamp to compare HGP in LDKO and LTKO mice. The glucose infusion rate (GIR), which is required to maintain euglycemia during insulin infusion (4 μM per kg per min), was significantly greater in Cntr mice than in LDKO mice (Fig. 1a); however, the GIR was normal in LTKO mice, despite the absence of hepatic Irs1 and Irs2 (Fig. 1b). Moreover, insulin failed to suppress HGP in LDKO mice, whereas HGP was suppressed normally by insulin in LTKO mice (Fig. 1c,d). Thus, hepatic insulin signaling was dispensable for glucose tolerance and the regulation of HGP when hepatic FoxO1 was inactivated.

**Hepatic FoxO1 dysregulates WAT insulin signaling.** Normal suppression of HGP in LTKO mice suggested that insulin indirectly suppressed HGP through its effects on WAT or other tissues. To investigate insulin signaling in the extrahepatic tissues of LDKO mice, we measured Akt phosphorylation on serine 473 and threonine 308 (pS473<sup>Akt</sup> and pT308<sup>Akt</sup>) in the skeletal muscle, epigonadal WAT (eWAT), inguinal WAT (iWAT) and brown adipose tissue (BAT) upon completion of the hyperinsulinemic-euglycemic clamp. Compared with Cntr mice, LDKO mice showed reduced levels of pS473<sup>Akt</sup> and pT308<sup>Akt</sup> in the eWAT, iWAT and BAT, with insignificant changes in skeletal muscle (Supplementary Fig. 1d–f). In contrast, pS473<sup>Akt</sup> and pT308<sup>Akt</sup> phosphorylation levels were normal in the eWAT and the iWAT isolated from LTKO mice (Supplementary Fig. 1g–i). Moreover, in contrast to Cntr mice, insulin-stimulated Irs1–p110<sup>PI3K</sup> complex formation was significantly decreased (by 49%) in the eWAT of LDKO mice, whereas the association was normal in the eWAT of LTKO mice (Fig. 1e). These results suggest that hepatic FoxO1 promoted WAT insulin resistance in LDKO mice.

To determine whether adult-onset hepatic insulin resistance promoted FoxO1-dependent WAT insulin resistance, LDKO mice were created acutely by infecting Irs1<sup>L/L</sup>; Irs2<sup>L/L</sup> mice with Cre<sup>ALb</sup>, the FDKO mice displayed normal glucose tolerance following acute Cre-mediated deletion of hepatic FoxO1 in the FDKO mice caused fasting hyperglycemia and severe glucose intolerance (Fig. 1h,i), suggesting that hepatic and adipose insulin resistance together synergistically disrupt glucose homeostasis (Supplementary Fig. 2j).

**Identification of FoxO1-dependent hepatokines in LDKO mice.** Previous work suggests that inflammatory cytokines might dysregulate systemic glucose metabolism<sup>6</sup>. We measured the circulating concentrations of several cytokines in LDKO and LTKO mice, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β, IL-2, IL-4, IL-5, chemokine (C-X-C motif) ligand 1 (CXCL1; also known as KC) and C-C motif chemokine ligand 3 (CCL3; also known as MIP1-α); however, only IL-6 displayed significant FoxO1-dependent elevation (Supplementary Fig. 3a–h). We generated IL-6<sup>-/-</sup> mice to test whether IL-6 contributed to glucose intolerance and WAT insulin resistance; however, glucose intolerance was not normalized in IL-6<sup>-/-</sup> mice compared to Cntr mice (Supplementary Fig. 3i). Furthermore, insulin-stimulated formation of the Irs1–p110<sup>PI3K</sup> complex remained low in the eWAT from IL-6<sup>-/-</sup> mice (Supplementary Fig. 3j). We conclude that the elevated levels of IL-6 were not responsible for the dysregulated glucose homeostasis and eWAT insulin resistance in LDKO mice.

To test whether serum from LDKO mice contains hepatic-derived circulating factors that modulate peripheral insulin action, cultured 3T3-L1 adipocytes were treated for 24h with 5% mouse serum obtained from LDKO or Cntr mice and then incubated briefly with insulin. Serum from the LDKO mice significantly decreased the phosphorylation of this list of secreted proteins that was confirmed by Ingenuity Pathway Analysis. This strategy revealed 19 putative hepatokines that were ≥3-fold higher in LDKO mice (Array Express accession no. E-MEXP-1649<sup>12</sup>). We further selected the genes that changed in LDKO mice but were expressed normally in LTKO mice and filtered this subset against a curated list of secreted proteins that was confirmed by Ingenuity Pathway Analysis. This strategy revealed 19 putative hepatokines that were ≥3-fold lower and 66 that were ≥3-fold higher in LDKO mice (Fig. 2a). Some of these hepatokines might have effects on peripheral metabolism—including lower levels of insulin-like...
growth factor-1 (Igf1; which mediates body growth), or higher levels of lipoprotein lipase (Lpl; which facilitates triglyceride uptake), follistatin (Fst; which inhibits TGF-β-Smad signaling) or insulin-like growth factor (Igf) binding protein 1 (Igfbp1; which inhibits Igf1 signaling). Thus, we infected wild-type (WT) mice maintained on a high-fat diet (HFD) with AAVTBG vectors encoding several candidate hepatokines or a control GFPAAV-TBG. The first cohort—including growth hormone receptor (Ghr), glutathione peroxidase 3 (Gpx3), serine peptidase inhibitor, clade B, member 6a (Serpinb6a), myelin protein zero-like 1 (Mplz1) or neurotrophin 3 (Ntf3)—had no effects on fasting glucose levels, insulin levels, glucose tolerance or insulin tolerance (Supplementary Fig. 4a–f).

In the second cohort, leukocyte elastase inhibitor A (Serpinb1a) and serum amyloid a4 (Saa4) decreased glucose levels without an effect on serum insulin levels, whereas Igf1 and inhibin E (Inhbe) increased serum insulin levels without effects on glucose levels (Fig. 2d,e). However, Fst315 (circulating Fst) increased circulating glucose and insulin levels, and the homeostatic model assessment of insulin resistance (HOMA-IR) suggested that Fst315 promoted systemic insulin resistance (Fig. 2d–f). Fst315 also promoted significant insulin resistance during the insulin tolerance test (Fig. 2f). Most of the tested hepatokines had an insignificant effect on glucose tolerance; however, Fst315 impaired glucose tolerance significantly, and Lpl significantly improved glucose tolerance (Fig. 2h,i). Finally, we confirmed the expression of the virus-encoded hepatokine genes in the liver by quantitative PCR (qPCR; Supplementary Fig. 4g,h). Fst315AAV-TBG infection of Cntr mice on a HFD increased circulating Fst315 levels by 23-fold (Fig. 2j).
Identification of Fst315 as a hepatic FoxO1-regulated hepatokine regulating systemic glucose homeostasis. a, Insulin-stimulated Irs1–p110PI3K complex formation in 3T3-L1 adipocytes cultured with serum from overnight-fasted 4-month-old LDKO (versus Cntr) mice without (left, n = 8) or with heat inactivation of the serum (56 °C for 45 min) (right, n = 4). b, c, Identified genes encoding putative hepatic secreted proteins that decreased (b) or increased (c) >3-fold in LDKO mice (Benjamini-Hochberg FDR < 0.05). Several of these secreted proteins that seemed likely to have effects on peripheral metabolism (marked with a black star) were selected for functional screening in mice. d–i, Five-week-old C57BL6 mice were challenged with a HFD (45% fat) for 2 months, then infected with GFPAAV-TBG or hepatokineAAV-TBG (2 × 10⁹ genome copy per mouse) encoding the indicated genes. Fasting blood glucose (d) and fasting serum insulin (e) were measured 1 week after infection and used to calculate HOMA2-IR (f) as a measure of systemic insulin resistance (n = 5–10). Insulin tolerance tests were performed 4 weeks after infection with GFPAAV-TBG or hepatokineAAV-TBG and summarized by the AUC (n = 4–10) (g). GTTs were performed 1 week after AAV infection (h) and summarized by the AUC (n = 4–10) (i). j, Fasting serum Fst levels measured 4 weeks after infection of HFD-fed C57BL6 mice with Fst315AAV-TBG or GFPAAV-TBG (n = 5). Data were analyzed by two-way ANOVA (h), one-way ANOVA (d–g) and unpaired Student’s t-test (a,j). Data are reported as the mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

To determine whether a more physiological 2-fold increase circulating Fst levels could dysregulate glucose tolerance, C57BL6 mice on a HFD for 2 months were infected with a lower dose of Fst315AAV-TBG or control GFPAAV-TBG. Ten days after Fst315AAV-TBG infection, circulating Fst levels were 2.2-fold higher than in controls, which still elevated fasting glucose levels and impaired glucose tolerance.

Fig. 2 | Identification of Fst315 as a hepatic FoxO1-regulated hepatokine regulating systemic glucose homeostasis. a, Insulin-stimulated Irs1–p110PI3K complex formation in 3T3-L1 adipocytes cultured with serum from overnight-fasted 4-month-old LDKO (versus Cntr) mice without (left, n = 8) or with heat inactivation of the serum (56 °C for 45 min) (right, n = 4). b, c, Identified genes encoding putative hepatic secreted proteins that decreased (b) or increased (c) >3-fold in LDKO mice (Benjamini-Hochberg FDR < 0.05). Several of these secreted proteins that seemed likely to have effects on peripheral metabolism (marked with a black star) were selected for functional screening in mice. d–i, Five-week-old C57BL6 mice were challenged with a HFD (45% fat) for 2 months, then infected with GFPAAV-TBG or hepatokineAAV-TBG (2 × 10⁹ genome copy per mouse) encoding the indicated genes. Fasting blood glucose (d) and fasting serum insulin (e) were measured 1 week after infection and used to calculate HOMA2-IR (f) as a measure of systemic insulin resistance (n = 5–10). Insulin tolerance tests were performed 4 weeks after infection with GFPAAV-TBG or hepatokineAAV-TBG and summarized by the AUC (n = 4–10) (g). GTTs were performed 1 week after AAV infection (h) and summarized by the AUC (n = 4–10) (i). j, Fasting serum Fst levels measured 4 weeks after infection of HFD-fed C57BL6 mice with Fst315AAV-TBG or GFPAAV-TBG (n = 5). Data were analyzed by two-way ANOVA (h), one-way ANOVA (d–g) and unpaired Student’s t-test (a,j). Data are reported as the mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

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FoxO1 regulates Fst expression in LDKO mice. Two Fst isoforms are generated by alternative mRNA splicing, including membrane-bound (autocrine) Fst288 and the longer circulating (endocrine) Fst315 (ref. 23). Compared to fed Cntr mice, hepatic FoxO1 mRNA (total and Fst315) and circulating Fst protein levels were 2-fold higher during fasting and 6-fold higher during streptozotocin-induced type 1 diabetes (Supplementary Fig. 5a–e). This pattern of expression was consistent with previous work showing that hepatic Fst is upregulated by an elevated glucagon/insulin ratio23. Although this ratio was relatively low in LDKO mice—glucagon levels were elevated 4-fold, whereas insulin levels were elevated 20-fold (Supplementary Fig. 5f,g)—the LDKO liver fails to respond to insulin, so the effect of glucagon might be exaggerated. Compared to the Cntr liver, the levels of mRNA in the LDKO liver encoding Fst288 and Fst315 increased 10-fold and 30-fold, respectively (Fig. 3a). Moreover, the concentration of circulating Fst in fasted LDKO mice increased 20-fold, and immunostaining revealed more Fst protein in liver sections (Fig. 3b,c). Fst mRNA levels were also higher in the liver of hepatocyte-specific insulin receptor knockout mice (LIRKO mice), confirming that hepatic insulin signaling suppressed Fst expression (Supplementary Fig. 5h). Consistent with these results, serum Fst levels were ~2-fold higher in genetically obese mice (ob/ob) or in Cntr mice fed a HFD (Supplementary Fig. 5i), which can be expected to impair glucose tolerance (Supplementary Fig. 4j).

Circulating and hepatic Fst levels were normal in the LTKO mice, suggesting that FoxO1 promoted its expression during hepatic insulin resistance (Fig. 3a,b). Moreover, injection of primary hepatocytes from C57BL6 mice with shFoxO1 decreased the levels of glucagon-stimulated Fst, compatible with a role for FoxO1 in stimulation by a high glucagon/insulin ratio (Fig. 3d). Consistent with these results, FoxO1 increased Fst mRNA levels and protein concentration in cultured hepatocytes and increased the concentration of Fst secreted into the medium (Fig. 3e). By contrast, infection with shFoxO1 decreased Fst mRNA levels and the concentration of Fst protein in cultured hepatocytes and in the medium (Fig. 3f).

Finally, we used chromatin immunoprecipitation (ChIP) with a FoxO1 antibody to determine whether FoxO1 binds directly to the Fst promoter. By comparison with strong FoxO1 binding to the Ppk1 promoter (positive control) or weak background binding to the Gapdh promoter (negative control), FoxO1 bound significantly between −525 and −2,175 of the Fst promoter (Fig. 3g). These regions contain several consensus FoxO1-binding sites that are homologous among human, mouse and rat, supporting the hypothesis that nuclear FoxO1 is an important regulator of Fst expression (Supplementary Fig. 5j).

Bariatric surgery reduces Fst in obese individuals with diabetes. Previous reports show that plasma Fst levels are elevated in individuals with T2D25–28. To investigate the relationship between glucose dysregulation and Fst in humans, we measured circulating Fst concentration in a group of obese individuals with diabetes (body mass index: 45.5 ± 3, hemoglobin A1c (HbA1c (%): 8.4 ± 1) before and after treatment by Roux-en-Y gastric bypass surgery (RYGB) (Supplementary Table 2).

Six months after RYGB, the HbA1c percentage decreased significantly to 6%, confirming a beneficial effect on glycemic control (Fig. 3h). Moreover, the serum Fst concentration correlated positively with the declining HbA1c percentage (Pearson correlation: \( r = 0.5977, P = 0.0088 \)), as it decreased significantly, by 48%, 6 months after RYGB (Fig. 3i). Importantly, the Fst concentration changed within the biologically active range (Supplementary Fig. 4i–k).

Thus, the beneficial effect of RYGB might be related, at least in part, to reduced circulating Fst levels.

Knockdown of hepatic Fst promotes WAT insulin sensitivity and reduces HGP. To establish whether excess endogenous hepatic Fst promotes WAT insulin resistance, we deleted hepatic Fst alleles by infecting the LDKO liver with clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9)-based AAV bearing two different guide RNAs targeting Fst (designated sgFst1AAV-TBG or sgFst2AAV-TBG). Compared to the control nullAAV-TBG vector, sgFst2AAV-TBG significantly reduced total hepatic and circulating Fst levels by 2-fold, whereas sgFst1AAV-TBG produced an intermediate effect that did not reach significance (Fig. 4a,b). Consistent with the differential reduction of Fst levels in the liver using these two different guide RNAs, glucose tolerance reached the normal range in LDKO sgFst2AAV-TBG mice, whereas LDKO sgFst1AAV-TBG mice displayed an intermediate but insignificant reduction (Fig. 4c). A similar differential effect on serum insulin levels was also observed with the two guide RNAs versus control (Fig. 4d).

Compared to nullAAV-TBG mice, the eWAT from LDKO sgFst2AAV-TBG mice displayed greater IRS1–p110PI3K complex formation and Akt phosphorylation (pT308Akt and pS473Akt) (Fig. 4e–g); however, pS660HSL levels were significantly lower in both LDKO sgFst1AAV-TBG mice and LDKO sgFst2AAV-TBG mice than in control (Fig. 4f,g).

The intermediate response to sgFst1AAV-TBG injection seems to arise from its variable infection or effect on Fst levels. To confirm the relationship between circulating Fst and the biological responses, we calculated Pearson correlation coefficients for the combined groups of mice. Significant correlations were calculated in all cases: Fst versus fasting insulin: \( r = 0.93, P < 0.0001; \) Fst versus GTT-AUC (area under the curve): \( r = 0.763, P < 0.0001; \) and Fst versus P30K: \( r = -0.408, P = 0.0310. \) Thus, the Fst concentration measured across each group (Cntr, null, sgFst1 and sgFst2) correlated significantly with each biological response. Taken together, these results suggest that lowering hepatic Fst levels improved insulin sensitivity of the eWAT and glucose tolerance in LDKO mice.

As CRISPR-Cas9-based gene editing can produce off-target effects, we used hepatic shFstAAV-TBG infection to validate the benefit of reducing hepatic Fst mRNA levels in LDKO mice (Supplementary Fig. 6a). Compared to the nullAAV-TBG infection, the eWAT from LDKO shFstAAV-TBG mice displayed significantly reduced levels of hepatic and circulating Fst (Supplementary Fig. 6b,c), improved glucose tolerance (Supplementary Fig. 6d), increased Akt phosphorylation (pT308Akt and pS473Akt) and decreased pS660HSL levels (Supplementary Fig. 6e,f). Together, these results confirm that hepatic-derived Fst promotes glucose intolerance and insulin resistance of the eWAT in LDKO mice.

To establish the relationship between hepatic Fst and HGP, the hyperinsulinemic-euglycemic clamp was conducted on LDKO mice infected with shFstAAV-TBG or nullAAV-TBG. The GIR required to maintain euglycemia was significantly greater in LDKO shFstAAV-TBG mice than in LDKO nullAAV-TBG mice, suggesting that reducing hepatic Fst levels increased systemic insulin sensitivity (Fig. 4h,i). Compared to the control LDKO nullAAV-TBG mice, HGP decreased in LDKO shFstAAV-TBG mice during the insulin clamp and the suppression of HGP by insulin was restored (Fig. 4j,k). Thus, knockdown of hepatic Fst in LDKO mice provided a benefit similar to that conferred by genetic disruption of hepatic FoxO1.

To establish whether Fst contributes to glucose intolerance in a non-genetic model of diabetes, C57BL6 mice maintained on a HFD for 2 months were infected with shFstAAV-TBG or nullAAV-TBG. Serum Fst levels were 37% lower in shFstAAV-TBG mice than in the control nullAAV-TBG mice (Fig. 4l). Suppression of hepatic Fst resulted in significantly lower circulating glucose levels before and during the GTT than in control-treated mice (Fig. 4m,n). Thus, hepatic Fst promoted glucose intolerance during genetic or physiological diabetes.
Fst promotes WAT insulin resistance and HGP in WT mice. Next, we investigated directly whether circulating Fst in LDKO mice can dysregulate adipose insulin signaling. Cultured 3T3-L1 adipocytes were incubated with mouse serum obtained from Cntr or LDKO mice, then treated briefly with insulin. Compared with serum from Cntr mice, serum from LDKO mice resulted in significantly lower (by 37%) insulin-stimulated Irs1–p110PI3K complex formation; moreover, the addition of an antibody to neutralize Fst in the LDKO serum resulted in greater Irs1–p110PI3K complex formation that approached the normal range (Fig. 5a).

To test whether Fst315 induced WAT insulin resistance in WT mice, C57BL6 mice maintained on a HFD for 4 months were infected with control GFPAAV-TBG or Fst315AAV-TBG. C57BL6 Fst315AAV-TBG mice displayed less 2-DOG ([1-14C] 2-deoxy-d-glucose) uptake (as a measure of insulin action) into the eWAT, iWAT and skeletal muscle upon completion of the hyperinsulinemic-euglycemic
**Fig. 4 | Knockdown of Fst improves WAT insulin sensitivity, reduces HGP and improves glucose tolerance in LDKO mice.**

a–g. Two-to-three-month-old LDKO mice were infected with nullAAV-TBG (encoding luciferase) or with sgFstAAV-TBG (encoding sgFst1 or sgFst2) to effect partial hepatic Fst knockout (n = 7). Hepatic Fst protein content (a) and serum total Fst concentration (b) were measured 2 weeks after infection. GTTs were performed 10 days after infection and summarized by the AUC (c). Fasting serum insulin levels were measured 2 weeks after infection (d). Insulin-stimulated Irs1–p110 PI3K complex formation in eWAT, measured 2 weeks after infection (e). Insulin-regulated phosphorylation of Akt and HSL in eWAT was analyzed 2 weeks after infection by immunoblotting (f) and quantitated by densitometry (g).

h–k. Five-month-old LDKO mice were infected with nullAAV-TBG or shFstAAV-TBG (encoding a microRNA-like hairpin RNA against Fst) and hyperinsulinemic-euglycemic clamps were conducted 4 weeks later (n = 5). Blood glucose concentrations (h) and GIRs (i) before and at steady state during the hyperinsulinemic-euglycemic clamp. Basal and clamped HGP (j) and the calculated suppression of HGP by insulin during the clamp (k).

l–n. Five-week-old C57BL6 mice maintained on a HFD for 2 months were infected with shFstAAV-TBG or nullAAV-TBG (n = 7). Serum Fst levels were measured 2 weeks after infection (l); GTTs (m) and fasting blood glucose levels (n) were measured 10 days after infection. Data (b,d,e) were reported as the median ± 95% CI and the Kruskal–Wallis test was used to identify significant differences between treatments of LDKO mice. Other data are reported as the mean ± s.e.m. Data were analyzed by one-way ANOVA (a,c,g), two-way ANOVA (i) and unpaired Student’s t-test (k–n). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
Adipocytes treated with serum from overnight-fasted 4-month-old LDKO (versus Cntr) mice, with or without the addition of a Fst antibody (10 μg/ml, AF669, R&D) (Fig. 5f, g). The change in serum FFA levels (the percentage of the overnight-fasted baseline) following insulin treatment (1 U per kg, intraperitoneal) of 14-week-old C57BL6 mice (maintained n=6). Basal and clamped HGP (the percentage of the overnight-fasted baseline) following insulin treatment (1 U per kg, intraperitoneal) of 4-month-old LTKO mice 2 weeks after infection with Fst315AAV-TBG or GFPAAV-TBG (n=10). Data in panel a were analyzed by ANOVA and compared by Benjamini-Hochberg FDR (*p < 0.05); other data were analyzed by unpaired Student’s t-test (b–e,g,k,n), one-way ANOVA (j,o) and two-way ANOVA (i,l,m). Data are reported as the mean ± s.e.m. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Fig. 5 | Fst315 promotes WAT insulin resistance, HGP and glucose intolerance in WT mice. a. Insulin-stimulated Irs1–p110PI3K complex formation in 3T3-L1 adipocytes treated with serum from overnight-fasted 4-month-old LDKO (versus Cntr) mice, with or without the addition of a Fst antibody (10 μg/ml, AF669, R&D) (n = 5–6). b–k. Five-week-old C57BL6 mice were challenged with a HFD for 4 months before infection with Fst315AAV-TBG or GFPAAV-TBG, and hyperinsulinemic-euglycemic clamps were performed 4 weeks later. Uptake of 2-DOG tracer into eWAT upon completion of the clamp. Blood glucose concentrations (h) and GIRs (i) before and during the hyperinsulinemic-euglycemic clamp (Fig. 5b–d); however, uptake by BAT was not affected (Fig. 5e). Both pT308Akt and pS473Akt levels were significantly higher, whereas pS660HSL levels were significantly lower in the eWAT of C57BL6 Fst315AAV-TBG mice (Fig. 5f,g). Unlike Fst315, Fst288 has a functional heparin-binding domain that can anchor it to the cell membrane upon secretion, where it might compete with receptors for binding to TGF-β superfamily ligands. We placed C57BL6 mice on a HFD for 2 months and then infected them with...
Fst288AAV−TRG. Four weeks later, hepatic Fst288 mRNA and protein levels were 128-fold and 4-fold higher, respectively, than in control mice (Supplementary Fig. 7a,b). Unexpectedly, circulating Fst levels were elevated by 12-fold 1 week after infection, indicating that hepatic Fst288 entered the circulation (Supplementary Fig. 7c). Compared to control C57BL6 GFPAAV−TRG mice on the HFD, fasting plasma insulin and blood glucose concentrations increased significantly in C57BL6 Fst288AAV−TRG mice (Supplementary Fig. 7d,e), which also developed glucose intolerance (Supplementary Fig. 7f). Fst288AAV−TRG infection resulted in a 2-fold lowering of 2-DG uptake into eWAT and iWAT upon completion of the hyperinsulinemic-euglycemic clamp (Supplementary Fig. 7g,h).

We next conducted hyperinsulinemic-euglycemic clamps to measure HGP in C57BL6 mice infected with GFPAAV−TRG, Fst315AAV−TRG or Fst288AAV−TRG. On a HFD, the GIR required to maintain euglycemia during the clamp in the GFPAAV−TRG-infected mice was significantly higher than in either Fst315AAV−TRG or Fst288AAV−TRG mice (Fig. 5h,i and Supplementary Fig. 7j,k,l). Moreover, the hepatic expression of either Fst315 or Fst288 increased HGP during basal and clamp conditions, and strongly reduced the ability of insulin to suppress HGP (Fig. 5j,k and Supplementary Fig. 7l). Compared to fasted Cntr mice on the HFD, insulin failed to suppress circulating FFAs in fasted C57BL6 Fst315AAV−TRG mice maintained on a HFD (Fig. 5i). To confirm the inhibitory effect of Fst on the suppression of lipolysis, metabolically normal LTKO mice were infected with Fst315AAV−TRG or control GFPAAV−TRG. Two weeks later, FFA levels after insulin treatment (1 U per kg) decreased normally in the Cntr mice, but only weakly in LTKO Fst315AAV−TRG mice (Fig. 5m). As in LDKO mice, urinary ketone levels were greater in the LTKO Fst315AAV−TRG mice than in the Cntr mice (Fig. 5n,o). These results are consistent with un-suppressed lipolysis owing to Fst-induced WAT insulin resistance.

**Regulation of hepatic gene expression by Fst depends partially on hepatic FoxO1.** To investigate how Fst affects hepatocytes per se, we used Affymetrix microarrays to quantify liver mRNAs from fasted mice infected with Fst288AAV−TRG or GFPAAV−TRG (Supplementary Fig. 8a). Fst288AAV−TRG infection modestly upregulated or downregulated the expression of many genes, including several genes related to hepatic glucose metabolism (Pck1, G6pc and Gck) (Supplementary Fig. 8a), and qPCR confirmed that Fst288AAV−TRG affected the expression of these and other genes during both fasting (Pck1, G6pc and Gck) and feeding (Foxo1, Igfbp1, Pck1 and Ppargc1a) (Fig. 6a,b). We also found that Fst288 expression was associated with a significant increase in glucose-6-phosphatase enzyme activity (encoded by G6pc) during fasting, which is essential for increased HGP (Fig. 6c). Microarray analysis revealed that Fst288 modestly downregulated several lipid and cholesterol synthetic genes (Supplementary Fig. 8a). We used qPCR to confirm that Fst288 reduced the expression of hepatic Srebf1, Fasn and Hmgcs1 (Supplementary Fig. 8b); moreover, immunoblotting showed that Fst288AAV−TRG increased HSL and decreased Srebp1 protein levels in the C57BL6 mouse liver (Supplementary Fig. 8c).

Hepatic FoxO1 mediates many effects of insulin on glucose and lipid metabolism11. To determine whether Fst288 modulates FoxO1 cellular distribution, we compared the concentration of FoxO1 in C57BL6 mice in nuclear and cytoplasmic fractions of liver lysates from fasting and fed C57BL6 Fst288 mice. Compared to control C57BL6 GFP mice, the total FoxO1 protein concentration was ~2-fold greater in the liver of C57BL6 Fst288 mice (Fig. 6d). Both nuclear and cytoplasmic FoxO1 concentrations were greater during fasting; however, during feeding, nuclear FoxO1 levels were greater, whereas cytoplasmic FoxO1 levels were lower, opposite to the regulation of FoxO1 by insulin (Fig. 6d).

To determine whether Fst288-regulated hepatic gene expression is dependent on FoxO1, we infected LTKO mice with Fst288AAV−TRG. RNA expression profiles showed that Fst288 regulated the expression of genes involved in gluconeogenesis and glycolysis, decreasing the expression of Gck and increasing the expression of Pck1 and G6pc, even without hepatic FoxO1; however, Fst288-mediated downregulation of lipogenic and cholesterogenic genes, such as Srebf1, was lost in the LTKO mice (Supplementary Fig. 8a). Next, to determine whether Fst288 required hepatic FoxO1 to promote HGP, GTTs and hyperinsulinemic-euglycemic clamps were performed. Compared to control GFPAAV−TRG LTKO mice, glucose tolerance was impaired mildly in Fst288AAV−TRG LTKO mice (Fig. 6e). The GIR required to maintain euglycemia during the clamp was also much less in Fst288AAV−TRG LTKO mice than in GFPAAV−TRG LTKO mice (Fig. 6f,g). As in LDKO mice, insulin failed to suppress HGP in the Fst288AAV−TRG LTKO mice (Fig. 6h,i); thus, the detrimental effect of Fst288 on HGP developed without hepatic FoxO1.

**Fst-mediated WAT insulin resistance is independent of hepatic FoxO1.** Finally, we investigated whether reduced Fst levels in LTKO mice contributed to their improved glucose tolerance and eWAT insulin sensitivity. We infected LTKO mice with Fst315AAV−TRG to increase circulating Fst315 levels by about ninefold (Supplementary Fig. 9a). Compared to control GFPAAV−TRG LTKO mice, the LTKO Fst315AAV−TRG mice developed profound glucose intolerance that phenocopied LDKO mice (Supplementary Fig. 9b). During insulin stimulation, both pT308−Akt and pS473−Akt levels decreased significantly—and pS660−Akt levels increased significantly—in the eWAT of LTKO Fst315AAV−TRG mice (Supplementary Fig. 9c,d). These results suggested that Fst315 promotes WAT insulin resistance independently of hepatic FoxO1. Moreover, the GIR required to achieve euglycemia decreased in clamped LTKO Fst315AAV−TRG mice (Supplementary Fig. 9e,f), and insulin failed to suppress HGP in LTKO Fst315AAV−TRG mice compared to LTKO GFPAAV−TRG mice (Supplementary Fig. 9g,h). Thus, FoxO1-dependent glucose dysregulation in LDKO mice is due, at least in part, to FoxO1-dependent upregulation of Fst, rather than hepatic FoxO1 alone.

**Discussion**

Our results identify the hepatokine Fst as a mediator of systemic metabolic dysregulation driven by hepatic FoxO1 activity. Previous investigations have highlighted the FoxO1-dependent nature of excessive HGP and/or glucose intolerance in mice with hepatic insulin resistance12,15,18. We previously showed that insulin fails to suppress HGP in our LDKO mice14; herein, we confirm that HGP suppression is restored in LDKO mice by disruption of hepatic FoxO1 (LTKO mice). Correlating with HGP, the expression and secretion of Fst are strongly upregulated in the LDKO liver but are nearly normalized in the LTKO liver. Within primary hepatocytes, knockdown of FoxO1 decreases—and its overexpression increases—the expression and secretion of Fst. Comparable with the direct regulation by FoxO1, we observe enriched recovery of Fst promoter regions containing FoxO1-binding motifs in anti-FoxO1 chromatin immunoprecipitates. Importantly, reducing hepatic production of Fst in LDKO mice using CRISPR–Cas9 or Fst shRNA mimics the benefits of Foxo1 deletion in LTKO mice. Conversely, viral expression of Fst in the liver of high-fat-fed WT mice or Chow-fed LTKO mice reproduces an LDKO-like phenotype, including impaired WAT insulin sensitivity, unsuppressed HGP and severe glucose intolerance. Thus, reduced expression and secretion of Fst may largely explain the restoration of metabolic homeostasis in LTKO versus LDKO mice15. Nonetheless, we do not rule out that other liver-intrinsic factors might contribute, particularly as viral expression of Fst alters RNA expression profiles in the livers of both WT and LTKO mice.

Although HGP and glucose tolerance are normalized in LTKO mice, the IRS–PI3K–Akt cascade in the liver of LTKO mice remains insensitive to insulin12. Our data support that normalization of
HGP in LTKO mice (and similar models) owes substantially to the restoration of WAT insulin sensitivity, which reduces lipolysis and the availability of FFAs and glycerol that potentiate hepatic gluconeogenesis\(^1,16,17\). Demonstrating the negative influence of hepatic FoxO1 on WAT insulin sensitivity, we observed significantly impaired IRK–IRS–Akt signaling and Akt–PDE3B–HSL signaling in the LDKO WAT, as well as impaired insulin suppression of serum FFAs—each of which is normalized in LTKO mice.

**Fig. 6** | Regulation of hepatic gene expression by Fst depends partially on hepatic FoxO1. a–d. C57BL6 mice challenged with a HFD for 2 months were infected with Fst288\(^{AAV-TBG}\) or GFP\(^{AAV-TBG}\). Hepatic mRNA expression was measured by qPCR after a 16-h overnight fast without (a) or with 4-h refeeding (b) 1 week after AAV infection. Glucose-6-phosphatase (G6PC) activity after a 16-h overnight fast in the liver 1 week after infection with Fst288\(^{AAV-TBG}\) or GFP\(^{AAV-TBG}\) (\(n = 5\)) (c). Western blot analysis and densitometric quantitation of FoxO1 protein in nuclear and cytoplasmic fractions or in the total liver lysate of C57BL6 mice, with or without 4-h refeeding following a 16-h overnight fast, and 1 week after infection with Fst288\(^{AAV-TBG}\) or GFP\(^{AAV-TBG}\) (\(n = 5\)) (d). e–i. Two-month-old LTKO mice were infected with GFP\(^{AAV-TBG}\) or Fst288\(^{AAV-TBG}\) to reconstitute hepatic Fst. GTTs were performed 2 weeks after AAV infection (\(n = 7\)) (e). Hyperinsulinemic-euglycemic clamps were conducted 4 weeks after infection; shown are the blood glucose concentrations (f) and the GIRs before and at steady state (g) during the hyperinsulinemic-euglycemic clamp (\(n = 5\)). Basal and clamped HGP (h) and the calculated suppression of HGP by insulin during the clamp (i) (\(n = 5\)). Data were analyzed by unpaired Student’s t-test (a–e, g, i) and one-way ANOVA (h). Data are reported as the mean ± s.e.m. *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\); ****\(P < 0.0001\).
Within the insulin-resistant liver, excess FFAs are oxidized to acetyl-CoA and further metabolized to produce ketone bodies. Our observation of elevated levels of urinary ketones in LDKO mice—but not LTKO mice—validates the oxidation of excess FFAs in the LDKO liver to acetyl-CoA, which allosterically upregulates pyruvate carboxylase-dependent hepatic gluconeogenesis and HGP. Forced expression of Fst in the LTKO liver likewise increases ketone production, in conjunction with impaired suppression of HGP and glucose intolerance. Together, these data support that WAT insulin resistance mediated by Fst contributes significantly to glucose intolerance in LDKO mice; however, additional secreted or non-secreted hepatic gene products regulated by FoxO1 might combine with Fst to produce the systemic effects of severe hepatic insulin resistance (for example, patatin-like phospholipase domain containing 2 (Pnpla2) and G0/G1 switch 2 (G0S2)).

Two Fst isoforms are produced by alternative mRNA splicing: membrane-bound (autocrine/paracrine) Fst288, which contains a functional heparin-binding site, and the longer circulating Fst315, which does not. Although our assays distinguish the hepatic mRNAs encoding these isoforms, available immunoassays cannot distinguish the protein isoforms. Both Fst288 and Fst315 mRNAs are upregulated in LDKO versus control or LTKO mice. Although its heparin-binding domain reportedly anchors Fst288 to cell membranes where it is produced, injection with either Fst288 or Fst315 increased circulating Fst levels in LTKO mice that weakly expressed endogenous Fst; moreover, the two isoforms equivalently dysregulated WAT insulin sensitivity, HGP and glucose tolerance. Regardless, mice engineered to produce only Fst288—without circulating Fst315—were shown by others to have mildly decreased fasting glucose levels. We conclude that its overexpression in Fst288-infected mice confers hepatokine/endocrine function on normally autocrine/paracrine Fst288.

Fst was identified originally as a soluble protein in follicular fluid that suppresses the biosynthesis and release of follicle-stimulating hormone from the pituitary. However, Fst binds to and neutralizes diverse TGF-β superfamily ligands. TGF-β superfamily signaling is initiated by heterotetrameric receptor serine kinases, composed of ‘type II’ and ‘type I’ receptors that establish the binding specificity and selective phosphorylation of R-Smads. Smad2 and Smad3 are downstream of TGF-β–activin–Nodal receptors, whereas Smad1, Smad5 and Smad8 lie downstream of the bone morphogenetic protein (BMP)–growth differentiation factor (GDF) pathway. These phosphorylated Smads assemble with Smad4 to enter the nucleus and modulate gene expression. Circulating Fst isoforms bind primarily to activin A and activin B, myostatin (also known as GDF8), and with lower affinity to BMP4 and BMP11 (refs 27,34,35,36). Circulating Fst-like 3 (Fstl3) binds to the same ligands as Fst, and deletion of Fstl3 improves glucose tolerance in mice. Neutralization of activin B might be relevant for the production of WAT insulin resistance, as it has been shown to suppress lipolysis in mouse embryo fibroblast-derived adipocytes and human adipocytes. However, as Fst is an inhibitor of TGF-β superfamily ligands, its activity on cultured adipocytes might depend on the mix of TGF-β ligands present in the medium. Moreover, BMP4 was shown to suppress lipolysis in pre-adipocytes by inhibiting HSL. Neutralization of other ligands of the TGF-β superfamily receptor Acvr2b could also be involved, as a soluble form of this receptor enhances gluconeogenesis in insulin-resistant mice.

Future assessment of changes in WAT mRNA transcription caused by ectopic Fst and/or TGF-β superfamily ligands would aid in validating the mechanism by which Fst impairs WAT insulin sensitivity. Along these lines, we observed that virus-mediatated Fst288 expression significantly alters the pattern of hepatic gene expression in high-fat-fed WT mice. As Fst expression produces a different pattern of hepatic gene expression in the LTKO liver, we suggest, provisionally, that excess circulating Fst may amplify, or alter, FoxO1-dependent hepatic gene expression changes. Regardless, as viral expression of Fst does not require hepatic FoxO1 to produce an LDKO-like phenotype (that is, in LTKO mice), Fst-induced changes in hepatic gene expression might be less critical for systemic dysregulation than its ability to promote WAT insulin resistance.

Previously, it was reported that insulin-resistant individuals and those with T2D exhibit modestly increased circulating Fst, and that this increase correlates positively with the percentage of HbA1c, fasting blood glucose, and glucose excursion during oral GTT. Although we did not measure circulating Fst levels in healthy controls, we found that RYGB significantly reduced circulating Fst levels in obese individuals with diabetes, and that this reduction correlated with the reduced percentage of HbA1c, an indicator of improved medium-term glycemic control. Thus, an unknown therapeutic aspect of RYGB might be to reduce the effect of circulating Fst on WAT, thereby facilitating control of HGP and glycemia. Although other interpretations of these data are possible, our mouse data support the notion that targeting heptically produced and secreted Fst in diabetic adults might prove useful in restoring systemic metabolic homeostasis. Because of its clear relation to hepatic insulin resistance and possible pleiotropic effects, targeting excess circulating Fst might also prove more fruitful than attempts to upregulate TGF-β superfamily-mediated signals in the insulin-resistant WAT.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41591-018-0048-0.

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Author contributions
M.F.W. and R.T. designed the research direction. R.T. performed the majority of the experiments with specialized assistance from C.W., K.D.C., O.S., W.Q., Y.H., J.M., S.L., X.C.D., L.L., M.S. and N.S. All data were analyzed by R.T. and M.F.W. The manuscript was written by R.T. and M.F.W. with assistance from K.D.C.

Competing interests
M.F.W. is a scientific consultant for Houser Pharmaceutical Research Laboratories.

Additional information
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Methods

Mice. We generated the liver-specific Irs1 and Irs2 double knockout (LDKO) and the Irs1, Irs2 and FoxO1 triple knockout (LTKO) mice as previously described.6,7 We purchased C57BL6 mice (stock no. 000664), B6/129S2−Icr(/) mice (stock no. 000632), B6.129S2−Icr(/)Kopl(/) mice (stock no. 002630) and B6.129S2−Icr(/)Kopl(+/−) mice. We purchased C57BL6 mice (stock no. 000632), B6.129S2−Icr(/)Kopl(+/−) mice. All mice were housed in plastic clogs on a 12 h/12 h light–dark cycle with free access to water and food. We followed animal experiments according to all relevant ethical regulations, and protocols were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee.

Hyperinsulinemic-euglycemic clamp in conscious and unrestrained mice. Prior to the clamp experiment, one catheter was inserted into the right jugular vein for infusions. After 5–7 days of recovery, mice that had lost <10% of their preoperative weight were subjected to the clamp. On the day of the experiment, mice were deprived of food for 3.5 h at 8:00 am and then infused continuously with d-[3-3H]−glucose (PerkinElmer) (0.05 μCi min−1) at a rate of 1 μl min−1 for 1.5 h. After basal sampling from the tail vein, a 140-min hyperinsulinemic euglycemic clamp was conducted with a primed-continuous infusion of human regular insulin (4 mU per kg per min, Humulin, Eli Lilly) at a rate of 2 μl min−1 and continuous infusion with d-[3-3H]−glucose (PerkinElmer) (0.1 μCi min−1) at a rate of 2 μl min−1 throughout the clamp experiment. The insulin solutions were prepared with 3% BSA in 0.9% saline. Glucose (20%) was infused at variable rates as needed to maintain plasma glucose at ~130 mg dl−1 (except in Fig. 1d, f where in treatments glucose was infused at 400 mg dl−1). LDKO mice. All mice were housed in plastic clogs on a 12 h/12 h light–dark cycle with free access to water and food. We followed animal experiments according to all relevant ethical regulations, and protocols were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee.

Blood chemistry analysis. We used commercial ELISA kits to measure circulating insulin and Fst levels according to the manufacturer's instructions. The insulin kit (80-INS05SU-E01) was purchased from Alpco Inc. Fst protein levels were measured using a Polyclonal Follistatin ELISA kit (DEN00, R&D Systems; 22% cross-reactivity with mouse Fst) following the manufacturer’s protocols with some modification. In brief, we applied 150 μl mouse serum (150 μl versus 100 μl) and increased the incubation time (overnight versus 2 h). IL-6 protein levels were measured using Mouse IL-6 ELISA Kit (Thermo Scientific Pierce, EM226L). The glucose blood level was measured using a glucose meter ( Bayer Contour). Urine ketone bodies were measured using Commercial assay kits (415-73301 and 411-73401, Wako USA). Serum IL-1β, IL-2, IL-1, IL-5, KC and MIP1α concentrations were measured using Milliplex MAP mouse magnetic bead panel kit.

Primary hepatocytes isolation. Eight-to-ten-week-old mice were anesthetized by intraperitoneal injection of ketamine/xylazine (100 mg per kg and 10 mg per kg body weight). Following anesthesia, the abdominal cavity was opened and the liver washed with scissors and the vena cava and portal vein were located. A perfusion catheter was placed into the vena cava. Pre-warmed Liver Perfusion Medium (17701, Invitrogen) (37 °C) was delivered at 1.6 ml min−1 for 12 min using a peristaltic pump. An incision was made at the portal vein as an outlet for the perfusion solution. Immediately following the Liver Perfusion Medium, pre-warmed Liver Digest Medium (17703, Invitrogen) (57 °C) was delivered at 1.6 ml min−1 for 12 min using a peristaltic pump. At the end of the perfusion, the liver was dissected and transferred to a Petri dish on ice containing 10 ml L-15 medium (21083, Invitrogen) with 10% FBS. After washing three times with Hepatocyte Wash Buffer (17704, Invitrogen), the primary hepatocytes were resuspended in William’s E Medium (12551, Invitrogen) containing Pencoll beads (p4937, Sigma). After 4 h of incubation in William’s E Medium containing 10% FBS and penicillin–streptomycin, the unattached cells were removed and the dishes were washed with PBS and incubated with maintenance media (William’s E medium containing 100 μM DEX, insulin–transferrin–seleium, 2 mM glutamine and PenStrep).

3T3-L1 cell culture. 3T3-L1 pre-adipocytes obtained from a mycoplasma-free stock were cultured in DMEM/F12 with 10% BCS in 5% CO2. Two days post-confluence, cells were exposed to DMEM/10% FBS with isobutylmethylxanthine (0.5 mM), dexamethasone (1 μM) and insulin (5 μg ml−1). After 2 days, cells were maintained in DMEM/F10% BCS until ready for treatment at day 7. On day 9, cells were treated with insulin (10 nM) for 3 min after being maintained in DMEM/5% mouse serum for 24 h.

Luminex assays. As described previously8,9, the Irs1 capture antibody (rabbit monoclonal antibody 58-10C-31, catalogue number 05-7841, Millipore) was coupled to magnetic carboxylated microspheres. The p110 subunit of PI3K was detected with antibodies from Cell Signaling Technology (4249). For Luminex assays, cells lysates (10 μg) or mouse tissue lysates (80 μg) were diluted with Irs1 capture beads (4,000 beads per well) into a total volume of 50 μl of phosphoprotein detection wash buffer (Bio-Rad) and incubated overnight in 96-well round-bottom plates. After washing twice with the same buffer, the beads were incubated with 50 μl detection antibody for 1 h on a rotary plate shaker (80 r.p.m.). After removal of the biotinylated detection antibody, the beads were incubated with shaking in 25 μl of 1 μg ml−1 streptavidin-phycoerythrin (Prozyme) for 15 min. All solutions were then removed and beads were suspended in PBS–BN (Sigma) for analysis in a Luminex FlexMap 3D instrument.

Histological and immunohistochemical analyses. The liver and WAT were fixed in phosphate-buffered paraformaldehyde (10%) and embedded in paraffin. H&E staining was conducted by the Pathology Core in the Dana-Farber Harvard Cancer Center. Fst immunostaining was conducted as described previously.10 To prove Fst in the liver sections, tissues were deparaffinized in xylene and rehydrated in a series of alcohol/water mixtures. Antigen retrieval was performed in 10 mM sodium citrate, pH 6.0, for 10 min. Tissue sections were blocked in 10% normal goat serum, incubated with the primary antibodies (203131, Abcam) overnight at 4°C and with goat anti-rabbit secondary antibody (A11012, Molecular Probes) for 1 h at room temperature (25°C). Slide mounting was performed using Prolong Antifade Gold with DAPI (162731, Life Technologies). Composite images were created from a 10 × 10 array of adjacent non-overlapping ×10 magnification images taken using a Zeiss Axiovert LSM 510 microscope.

Nuclear and cytoplasmic protein extraction. The nuclear and cytoplasmic proteins were differentially extracted as directed using a kit from Thermo Scientific (NE-PER Nuclear and Cytoplasmic Extraction Kit, 78835).

G6Pase activity measurement. G6Pase activity assays were performed as previously described.11 Briefly, 100 μg liver was homogenized in buffer (HEPES, 5 mM; sucrose, 250 mM) using a dounce homogenizer. Microsomes were prepared using ultracentrifuge at 100,000 × g at 4°C for 60 min. Microsomes were performed using ultracentrifuge at 100,000 × g at 4°C for 60 min. Microsomes were mixed with the microsomal substrates (G6P, 1–5 mM; sodium cadoxalate, 183.3 mM; histidine type IIA, 10 mM)11. Absorbance was measured at 820 nm.
Bypass surgery samples information. Human serum used in this study was obtained as a part of a larger clinical study approved by both the Boston Children’s Hospital IRB (IRB-P00021478; principal investigator: N.S.) and the University of Pittsburgh Medical Center IRB (UPMC IRB MOD15099064-02/PR015099064). Informed and consenting individuals underwent RYGB by the Minimally Invasive Bariatric and General Surgery (MBGS) group of the UPMC. Blood was collected by venipuncture at a research visit prior to surgery (baseline) and 6 months after surgery. Individuals taking metformin or statins were asked to temporarily discontinue these treatments starting on the night before surgery. Diabetes was defined as either a documented fasting blood glucose level of >126 mg/dl or HbA1c ≥2.6%, or treatment with an anti-diabetic medication. The mean percentage of HbA1c in diabetic individuals was 8.35 ± 1.3.

AdV vector preparation. To make FoxO1 and GFP overexpression AdVs, mouse FoxO1 and GFP coding sequences were first subcloned into a pShuttle-IRES- herGFP-2 vector (Agilent) and then transferred to a pAAVEasy vector (Agilent), as described previously37. The constructs were digested by enzyme PacI (Ro547, NEB) and then transfected into human embryonic kidney (HEK) 293A cells for AdV production. To make shRNA Ad to knock down Foxo1, a gene-specific shRNA (top strand: 5′-GAGCGTGCCCTACTTCAAGGA) was designed using an online tool (BLOCK-iT, Life Technologies). The hairpin-encoding oligonucleotides were cloned in the vector pENTR/U6 (Life Technologies), which was recombined with pAd/BLOCK-iT vectors. The positive clones were used for transfection of HEK293A cells to make AdVs, as described previously40.

AAV vector preparation. AAV.TBG.plGFP (GFPatt, MSC: Lot: AV-8-PV0146), AAV.TBG.plCre (Creatt, MSC: Lot: AV-8-PV0191) and AAV.TBG.plNullGHiGH (nullatt, MSC: Lot: AV-8-PV0148) were purchased from the Vector Core, University of Pennsylvania. To make overexpression AAVs, mouse gene coding sequences were cloned into a pAAV backbone with the Tbg promoter and paired upstream of the alpha Tbg (indicated throughout) were chosen to equal to three or more for in vitro studies and seven or more for in vivo studies. The expression cassettes were then subcloned into the pX602-AAV-TBG::NLS-SaCas9-NLS-HA-OLLAS-bGHpA;U6::BsaI-sgRNA;P2A::foxo1-EGFP-2;U6::BsaI-sgRNA (Sigma) and then subjected to a clean-up step using an RNeasy Mini Kit with DNAse (Qiagen). Purified DNAs were amplified and quantified using Power SYBR Green PCR Master Mix (Life Technologies) and promoter-specific primers (Supplementary Table 5) in QuantStudio 6 Flex.

RNA extraction and qPCR analysis. RNA isolation was performed as described previously48. Real-time RT-PCR was performed in two steps. First, cDNA was synthesized using a cDNA synthesis kit (Bio-Rad). Second, cDNA was analyzed by real-time PCR using gene-specific primers (Supplementary Table 6) and SYBR Green Master Mix (Applied Biosystems).

Affymetrix MoGene-2.0-0st arrays. RNA was extracted using TRI reagent (Sigma) and then subjected to a clean-up step using an RNeasy Mini Kit with DNAse (Qiagen). Gene expression profiles were obtained using the Affymetrix MoGene-2.0-0st Chip at the Genetics Core facility of Boston Children's Hospital.

Statistical analysis. Two-tailed, unpaired Student’s t-tests were used to assess statistical significance between two groups. Multiple groups or treatments were compared using one-way analysis of variance (ANOVA), two-way ANOVA or Kruskal–Wallis test, as appropriate to the design and/or data distribution. When ANOVA indicated a significant difference among the groups, differences between groups were identified using a stricter criterion for significance according to the Bonferroni rule. Owing to the non-normal distribution of data in Fig. 4b,d,e, data are reported as the median ± 95% confidence interval (CI). No data were considered outliers by testing or were arbitrarily excluded. The sample sizes (indicated throughout) were chosen to equal to three or more for in vitro studies and five or more (for example, mice per group) for in vivo experiments. Data collection for screening of putative hepatokines (Fig. 2) in WT mice was performed in a blind manner and was unblinded for treatment (hepatokine). Arrays (Supplementary Figs. 2i and 8a) were performed by the Molecular Genetics Core at Boston Children’s Hospital and were blind to genotype. GraphPad Prism 7 software was used for all data analysis.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The Array Express accession number for data in Fig. 3b,c is E-MEXP-1649. The data in Supplementary Figs. 2i and 8a have been deposited in the NCBI's Gene Expression Omnibus and are accessible through the GEO Series accession number GSE111899.

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Experimental design

1. Sample size
   - Describe how sample size was determined.
   - The sample sizes (indicated throughout) were chosen to equal to three or greater for in vitro studies and five or greater (e.g. mice per group) for in vivo experiments.

2. Data exclusions
   - Describe any data exclusions.
   - Presented data, and statistical treatment, include the entirety of the planned experiments; no data were considered outliers by testing or arbitrarily excluded.

3. Replication
   - Describe whether the experimental findings were reliably reproduced.
   - Key findings/conclusions regarding peripheral insulin resistance in the LDKO model of hepatic insulin resistance, as well as regulation of follistatin by hepatic FoxO1 were reproduced/corroborated by contrasting the results of like assays in diverse genetic contexts, including:
     1) control (floxed Irs1/2) versus LDKO mice,
     2) LDKO versus LTKO (LDKO with hepatic FoxO1 knockout) mice,
     3) LTKO + adenoviral FoxO1 restoration,
     4) acutely generated LDKO mice (flox mice plus viral Cre) to examine developmental dependency
   - Key findings/conclusions concerning the role of follistatin in vivo were reproduced/corroborated by:
     1) Overexpression of Fst in wild-type (high fat-fed) mice
     2) Knockdown of hepatic follistatin expression in LDKO mice: two separate methods were used for knockdown (shFst and sgFst/CRISPR) with similar results. Additionally, the results of two sgFst sequences are shown.
     3) Knockdown of hepatic follistatin in mice maintained on high fat diet
     4) Viral reconstitution of follistatin expression in hepatocytes of LTKO mice
     5) Fst expression was checked/confirmed repeatedly at both hepatic message (mRNA) and secreted protein (serum) levels in the above models plus/minus the experimental manipulations.
   - Gene expression (increased or decreased) implied by transcriptomic approach (Affymetrix arrays) was confirmed by qPCR of follistatin and other mRNAs. In vivo overexpression (screening) of putative hepatokines was also confirmed by qPCR.

4. Randomization
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - Mice were randomly divided into control and treatment groups. All included human data are from treated individuals (bariatric surgery).

5. Blinding
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - Data collection for screening of putative hepatokines (Fig. 2) in WT mice was performed in blind fashion and unblinded for treatment (hepatokine). Microarray assays (Fig S8a-b) were performed by service for fee that was blind to genotype. Note: all human data herein come from subjects in the same (surgery) treatment group.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☑   | ☑         |

☑ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

☑ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☑ A statement indicating how many times each experiment was replicated

☑ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)

☑ A description of any assumptions or corrections, such as an adjustment for multiple comparisons

☑ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted

☑ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)

☑ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study. Alpha Innotech FluorChem 5500 was used to quantify the blots. GraphPad Prism 7 was used to perform analysis.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company. No unique materials as described under the policy information were used.
9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

| Antibodies | Source |
|------------|--------|
| Phospho-Akt (Ser473) | Cell Signaling Technology CST#4058 |
| Phospho-Akt (Thr308) | Cell Signaling Technology CST #4056 |
| Akt | Cell Signaling Technology CST #9272 |
| Phospho-HSL (Ser660) | Cell Signaling Technology CST #4126 |
| HSL | Cell Signaling Technology CST #4107 |
| FoxO1 | Cell Signaling Technology CST #2880 |
| β-Actin | Cell Signaling Technology CST #4967 |
| Phospho-Smad2 (Ser465/467) | Cell Signaling Technology CST #3101 |
| Smad2 (D43B4) | Cell Signaling Technology CST #5339 |
| GAPDH (D16H11) | Cell Signaling Technology CST #5174 |
| Lamin A/C | Cell Signaling Technology CST #2032 |
| Actinin | Santa Cruz Biotechnology SC-15335 |
| Irs1 mouse polyclonal | Produced in our laboratory |
| Irs2 mouse polyclonal | Produced in our laboratory |
| Srebp1 | Abcam #3259 |
| Prolong Antifade Gold with DAPI | Life Technologies #1652731 |
| Fst primary antibodies for cell culture | R&D Systems #AF669 |
| Goat anti-rabbit secondary antibody | Molecular Probes #A11012 |

Validation: Irs1 and Irs2 antibodies were validated using liver samples from Irs1/2-flox and Irs1/2-LDKO mice. Fst antibody was validated by correlation with viral overexpression of Fst in mouse liver. All others were used according to instruction of the products.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

| Cell Line | Source |
|-----------|--------|
| 3T3-L1 adipocytes | (gift of Dr. Evan Rosen’s lab) |
| HEK293 | ATCC CRL-1573 |
| 3T3-L1 adipocyte and HEK293 cells | were authenticated by STR Profiling. |

b. Describe the method of cell line authentication used.

| Method | Status |
|--------|--------|
| Both cell lines | were tested negative for mycoplasma contamination |
| No commonly misidentified cell lines | were used |

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

| Species | Source |
|---------|--------|
| C57BL6/J mice | The Jackson Laboratory #000664 |
| Ob/Ob mice | The Jackson Laboratory #000632 |
| B6;FVB-Tg(Adipoq-cre)1Evdr/J | The Jackson Laboratory #010803 |
| Irs1-flox | Generated in our laboratory |
| Irs2-flox | Generated in our laboratory |
| FoxO1-flox | From Ronald DePinho’s lab |
| B6.129S2-Ile6tm1Kopf/J | The Jackson Laboratory #002650 |
12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

Human serum samples: Human serum used in this study was obtained as a part of a larger clinical study approved by both the Boston Children’s Hospital IRB (IRB-P00021478; PI: Nicholas Stylopoulos) and the University of Pittsburgh Medical Center IRB (MOD15090464-02 / PRO15090464; PI: Anita Courcoulas). This is an ongoing, prospective, single-center study. Patients underwent RYGBS by the Minimally Invasive Bariatric and General Surgery (MIBGS) group of the University of Pittsburgh (UPMC). Blood was collected by venipuncture at a research visit prior to surgery (baseline) and 6 months after surgery. All bariatric surgeries were performed on a clinical basis and were not performed on a research basis. 15 of 18 patients, or 83.33% of participants, were female. Average baseline BMI was 45.29 ±7.29 kg/m² and did not differ between diabetic and non-diabetic patients. Patients taking Metformin or statins were asked to temporarily discontinue these medications starting on the night prior to surgery. Measurements were collected including weight, % body fat using the Tanita Scale, and the height using a stadiometer. Diabetes was defined as either a documented fasting blood glucose level >126 mg/dl or an HbA1c of 6.5% or higher, or treatment with an anti-diabetic medication. Average HbA1c in non-diabetic patients was 5.55±0.36% and, in diabetic patients, 8.35±1.26%. Detailed information provided in Table S2.