



# Forkhead box protein O1 (FoxO1) regulates hepatic serine protease inhibitor B1 (serpinB1) expression in a non-cell-autonomous fashion

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FoxO proteins are major targets of insulin action, and FoxO1 mediates the effects of insulin on hepatic glucose metabolism. We reported previously that serpinB1 is a liver-secreted factor (hepatokine) that promotes adaptive  $\beta$ -cell proliferation in response to insulin resistance in the liver-specific insulin receptor knockout (LIRKO) mouse. Here we report that FoxO1 plays a critical role in promoting serpinB1 expression in hepatic insulin resistance in a non-cell-autonomous manner. Mice lacking both the insulin receptor and FoxO1 (LIRFKO) exhibit reduced  $\beta$ -cell mass compared with LIRKO mice because of attenuation of  $\beta$ -cell proliferation. Although hepatic expression of serpinB1 mRNA and protein levels was increased in LIRKO mice, both the mRNA and protein levels returned to control levels in LIRFKO mice. Furthermore, liver-specific expression of constitutively active FoxO1 in transgenic mice induced an increase in hepatic serpinB1 mRNA and protein levels in refed mice. Conversely, serpinB1 mRNA and protein levels were reduced in mice lacking

FoxO proteins in the liver. ChIP studies demonstrated that FoxO1 binds to three distinct sites located  $\sim 9$  kb upstream of the *serpinb1* gene in primary mouse hepatocytes and that this binding is enhanced in hepatocytes from LIRKO mice. However, adenoviral expression of WT or constitutively active FoxO1 and insulin treatment are sufficient to regulate other FoxO1 target genes (IGFBP-1 and PEPCK) but not serpinB1 expression in mouse primary hepatocytes. These results indicate that liver FoxO1 promotes serpinB1 expression in hepatic insulin resistance and that non-cell-autonomous factors contribute to FoxO1-dependent effects on serpinB1 expression in the liver.

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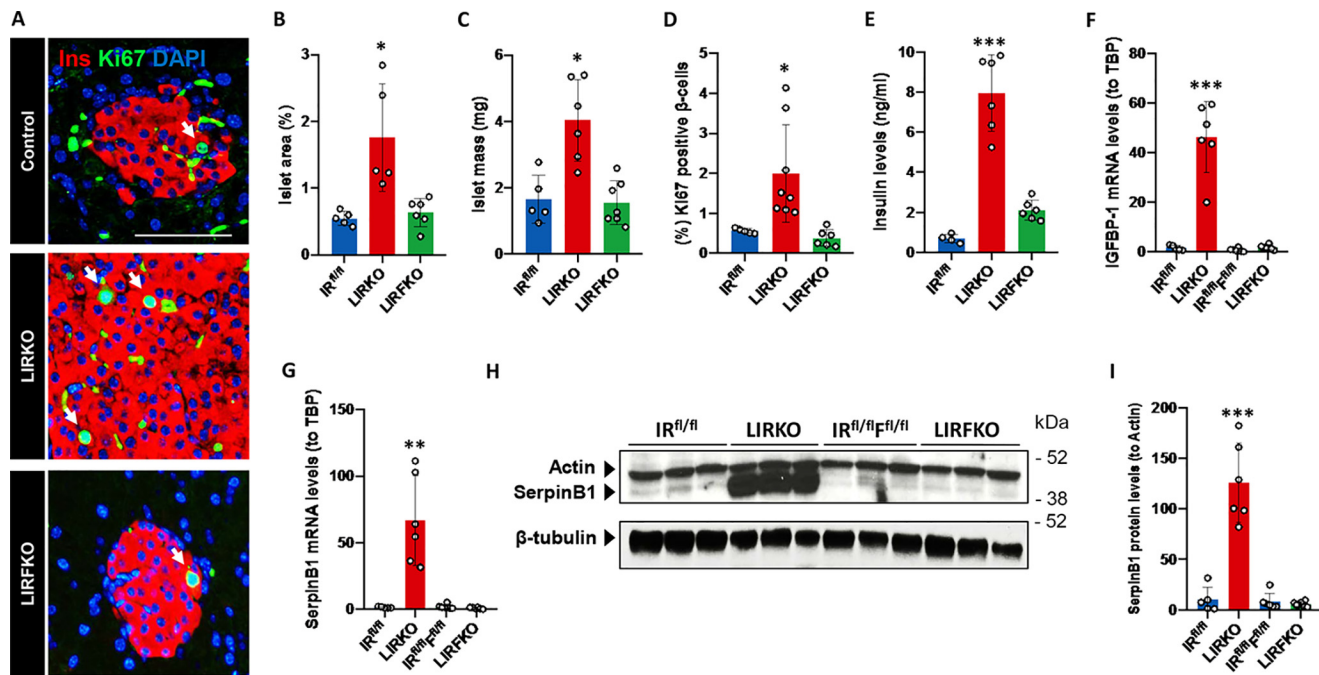
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Diabetes mellitus develops when the number or function of pancreatic  $\beta$ -cells is insufficient to maintain normoglycemia. Typically, the mass of insulin-producing  $\beta$ -cells is reduced in diabetes, including the most commonly occurring types in the general population: type 1 and type 2 diabetes (1–3). Efforts to identify the molecular and cellular mechanisms that enable regeneration of pancreatic  $\beta$ -cells continue to be a major focus of diabetes research (4–14). Competing strategies include the generation of insulin-secreting cells from pluripotent stem cells (9, 10, 15) or other sources (4, 8, 11, 13, 16) and the proliferation of pre-existing islet  $\beta$ -cells (5–7, 12, 17). The evolving stem cell-based approaches coupled with their oncogenic potential after transplantation suggest that this is not yet ready for therapeutic consideration (18). Current strategies have focused on harnessing the replicative activity (19, 20) of  $\beta$ -cells, which is known to increase significantly in response to challenges such as insulin resistance (17, 21–23). Thus the identification of endogenous molecules that selectively enhance human  $\beta$ -cell numbers have the potential to yield safe anti-diabetic medications (24).

We have recently reported that serpinB1, a liver-derived molecule, can function as a novel  $\beta$ -cell growth factor (6). Although serpinB1 is expressed at very low levels in the liver under normal conditions, hepatic serpinB1 expression and production are considerably enhanced in models of insulin



**Figure 1.  $\beta$ -Cell proliferation and expression of serpinB1 in the livers of LIRKO and LIRFKO mice.** *A*, representative images of insulin<sup>+</sup>/Ki67<sup>+</sup> cells (arrows) in pancreata isolated from 10-week-old male IR<sup>fl/fl</sup> (top panel), LIRKO (center panel), or LIRFKO (bottom panel) mice. Scale bar = 50  $\mu$ m. *B*, quantification of islet area. Data are presented as the percentage of total islet cross-sectional area and represent mean  $\pm$  S.E. \*,  $p \leq 0.05$  versus IR<sup>fl/fl</sup> ( $n = 5-8$ /group). *C*, quantification of islet mass. Data represent mean  $\pm$  S.E. \*,  $p \leq 0.05$  versus IR<sup>fl/fl</sup> ( $n = 5-8$ /group). *D*, quantification of insulin<sup>+</sup>/Ki67<sup>+</sup> cells (in *A*). Data represent mean  $\pm$  S.E. \*,  $p \leq 0.05$  ( $n = 5-8$ /group). *E*, insulin levels. Data represent mean  $\pm$  S.E. \*\*\*,  $p \leq 0.001$  ( $n = 4-6$ /group). *F*, relative liver *igfbp-1* mRNA levels by real-time PCR (normalized for TBP) in 10-week-old male IR<sup>fl/fl</sup>, IR<sup>fl/fl</sup>F<sup>fl/fl</sup>, LIRKO, and LIRFKO mice. Data represent mean  $\pm$  S.E. \*\*\*,  $p \leq 0.001$  ( $n = 5-6$ /group). *G*, relative liver *serpin1* mRNA levels in livers of 10-week-old male IR<sup>fl/fl</sup>, IR<sup>fl/fl</sup>F<sup>fl/fl</sup>, LIRKO, and LIRFKO mice. Data represent mean  $\pm$  S.E. \*\*,  $p \leq 0.01$  ( $n = 5-6$ /group). *H*, Western blots of serpinB1, actin, and  $\beta$ -tubulin in liver. *I*, quantification of serpinB1 protein. Data represent mean  $\pm$  S.E. \*\*\*,  $p \leq 0.001$  ( $n = 5-6$ /group).

resistance, including liver-specific insulin receptor knockout (LIRKO)<sup>7</sup> mice, a unique model of islet  $\beta$ -cell hyperplasia (17, 22). SerpinB1 also stimulates islet  $\beta$ -cell proliferation in multiple species, including zebrafish, mice, and humans, concomitant with activation of proteins in the growth factor signaling pathway (6).

FoxO Forkhead proteins are major targets of insulin action (25–28). Insulin activates Akt, which induces phosphorylation at three highly conserved sites corresponding to Thr-24, Ser-256, and Ser-319 in human FoxO1. Akt-dependent phosphorylation leads to the translocation of FoxO proteins from the nucleus and sequestration in the cytoplasmic compartment in association with 14-3-3 proteins (29, 30). Recent studies indicate that FoxO proteins promote hepatic glucose production by multiple mechanisms. In addition to enhancing the transcription of key enzymes encoded by phosphoenolpyruvate carboxykinase (*pepck*) and glucose 6-phosphatase (*g6pase*) and suppressing glycolytic and lipogenic gene expression and metabolism by direct mechanisms (30, 31), studies of LIRKO and IR/FoxO1 double knockout (LIRFKO) mice indicate that liver FoxO1 also impacts the ability of insulin to regulate hepatic glucose production through extrahepatic mechanisms, including effects on the ability of insulin to suppress lipolysis in

white adipose tissue (32), presumably because of production of FoxO1-dependent hepatokines.

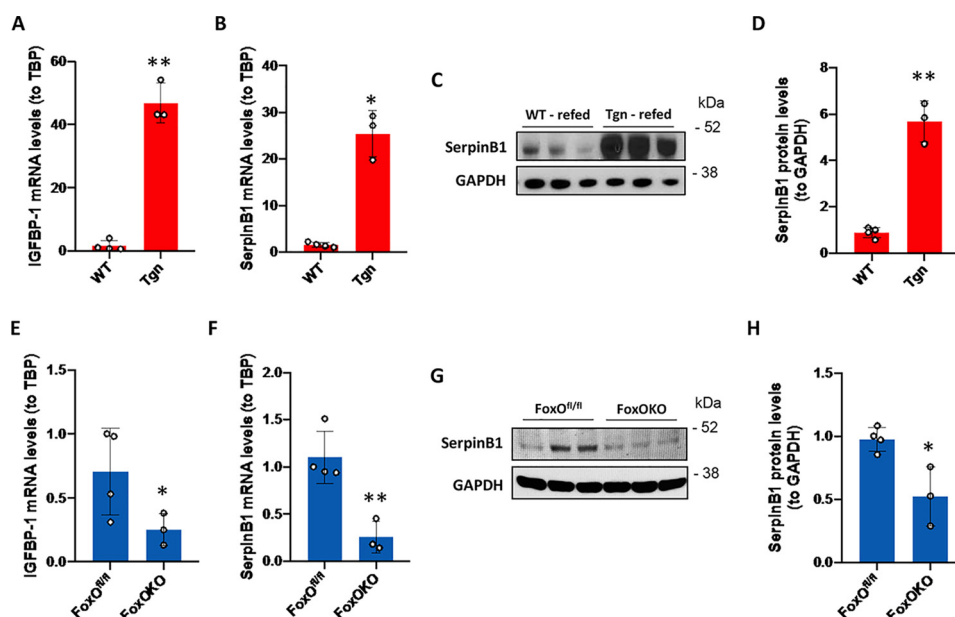
Previously, we reported that, in addition to restoring the ability of insulin to maintain glucose homeostasis and suppress hepatic glucose production, disrupting FoxO1 expression in the liver also reverses  $\beta$ -cell hyperplasia in LIRKO mice (25). Because serpinB1 plays an important role in mediating the effects of hepatic insulin resistance on  $\beta$ -cell proliferation (6), we asked whether FoxO1 contributes to the expression of serpinB1 in the liver of LIRKO mice and whether these effects are mediated through direct, intrahepatic mechanisms and/or whether non-cell-autonomous factors are involved. Here we report that hepatic FoxO1, a major target of insulin action in the liver (25), plays an important role in regulating the expression of serpinB1 in the setting of hepatic insulin resistance and that this effect may involve both direct and indirect mechanisms, similar to FoxO1 regulation of hepatic glucose production.

## Results

### $\beta$ -cell proliferation and hepatic serpinB1 expression in LIRKO versus LIRFKO mice

We first asked whether hepatic FoxO1 plays a critical role in promoting islet  $\beta$ -cell proliferation in the setting of hepatic insulin resistance using LIRKO and LIRFKO mice. We measured islet mass and  $\beta$ -cell replication in 10-week-old male insulin receptor–floxed (IR<sup>fl/fl</sup>), LIRKO, and LIRFKO mice by insulin and Ki67 co-immunostaining. As shown in Fig. 1, *A–D*, islet area, islet mass, and  $\beta$ -cell proliferation were increased 3–

<sup>7</sup> The abbreviations used are: LIRKO, liver-specific insulin receptor knockout; IR, insulin receptor; LIRFKO, liver-specific insulin receptor and FoxO1 knockout; Tgn, transgenic; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; m.o.i., multiplicity of infection; HGP, hepatic glucose production; WAT, white adipose tissue; qPCR, quantitative PCR; qRT-PCR, quantitative real-time PCR; TBP, TATA-binding protein.



**Figure 2. Regulation of hepatic serpinB1 by FoxO proteins *in vivo*.** A–D, 10-week-old male WT and Tgn mice were fasted overnight for 18 h and refed for 6 h before livers were harvested for analysis of mRNA and protein expression. A, relative liver *igfbp-1* mRNA levels (normalized to TBP) in 10-week-old WT and Tgn mice. Data represent mean  $\pm$  S.E. \*\*,  $p \leq 0.01$  ( $n = 3-4$ /group). B, relative liver *serpinb1* mRNA (normalized to TBP) in 10-week-old WT and Tgn mice. Data represent mean  $\pm$  S.E. \*,  $p \leq 0.05$  ( $n = 3-4$ /group). C, Western blot of serpinB1 and GAPDH in liver. D, quantification of serpinB1 protein. Data represent mean  $\pm$  S.E. \*\*,  $p \leq 0.01$  ( $n = 3-4$ /group). E–G, 10-week-old male FoxO<sup>fl/fl</sup> and FoxOKO mice were fasted overnight for 18 h prior to sacrifice, and then livers were harvested for quantification of gene and protein expression. E, relative liver *igfbp-1* mRNA levels by in (normalized to TBP) 10-week-old FoxO<sup>fl/fl</sup> and FoxOKO mice. Data represent mean  $\pm$  S.E. \*,  $p \leq 0.05$  ( $n = 3-4$ /group). F, relative liver *serpinb1* mRNA levels (normalized to TBP) in 10-week-old FoxO<sup>fl/fl</sup> and FoxOKO mice. Data represent mean  $\pm$  S.E. \*\*,  $p \leq 0.01$  ( $n = 3-4$ /group). G, Western blot of serpinB1 and GAPDH in liver. H, quantification of serpinB1 protein. Data represent mean  $\pm$  S.E. \*,  $p \leq 0.05$  ( $n = 3-4$ /group).

to 4-fold in LIRKO compared with IR<sup>fl/fl</sup> littermate controls, consistent with previous studies (6, 17, 22). In contrast, islet mass and  $\beta$ -cell proliferation were not increased in LIRFKO mice compared with floxed controls (Fig. 1, A–D), indicating that FoxO1 plays an important role in promoting  $\beta$ -cell proliferation in the setting of hepatic insulin resistance. In agreement with changes in islet  $\beta$ -cell proliferation and area, LIRKO mice exhibited 11-fold higher insulin levels compared with control mice, and this effect was also largely reversed in LIRFKO mice (Fig. 1E).

Next, we assessed the abundance of serpinB1 mRNA and protein levels in the liver of LIRKO, LIRFKO, and IR<sup>fl/fl</sup> mice. For comparison, we also assessed the expression of insulin-like growth factor–binding protein 1 (IGFBP-1), a canonical FoxO1 target gene that is directly regulated by FoxO proteins through well-characterized *cis*-acting FoxO-binding sites located within the proximal IGFBP-1 promoter (33). As expected, IGFBP-1 mRNA levels were increased ~50-fold in LIRKO livers compared with IR<sup>fl/fl</sup> or IR/FoxO1-floxed (IR<sup>fl/fl</sup>F<sup>fl/fl</sup>) mice, whereas IGFBP-1 mRNA levels were not increased in LIRFKO-derived livers relative to IR<sup>fl/fl</sup> and IR<sup>fl/fl</sup>F<sup>fl/fl</sup> mice (Fig. 1F). Gene expression studies showed that liver serpinB1 mRNA levels also were increased ~70-fold in LIRKO compared with IR<sup>fl/fl</sup> mice (Fig. 1G), consistent with previous studies (6). In contrast, LIRFKO mice displayed no increase in serpinB1 gene expression compared with floxed controls, indicating that FoxO1 plays a critical role in promoting the expression of serpinB1 in the liver when hepatic insulin signaling is disrupted, suggesting effects on gene transcription, although effects on pre-mRNA or mRNA stability cannot be ruled out. Similarly, Western blot-

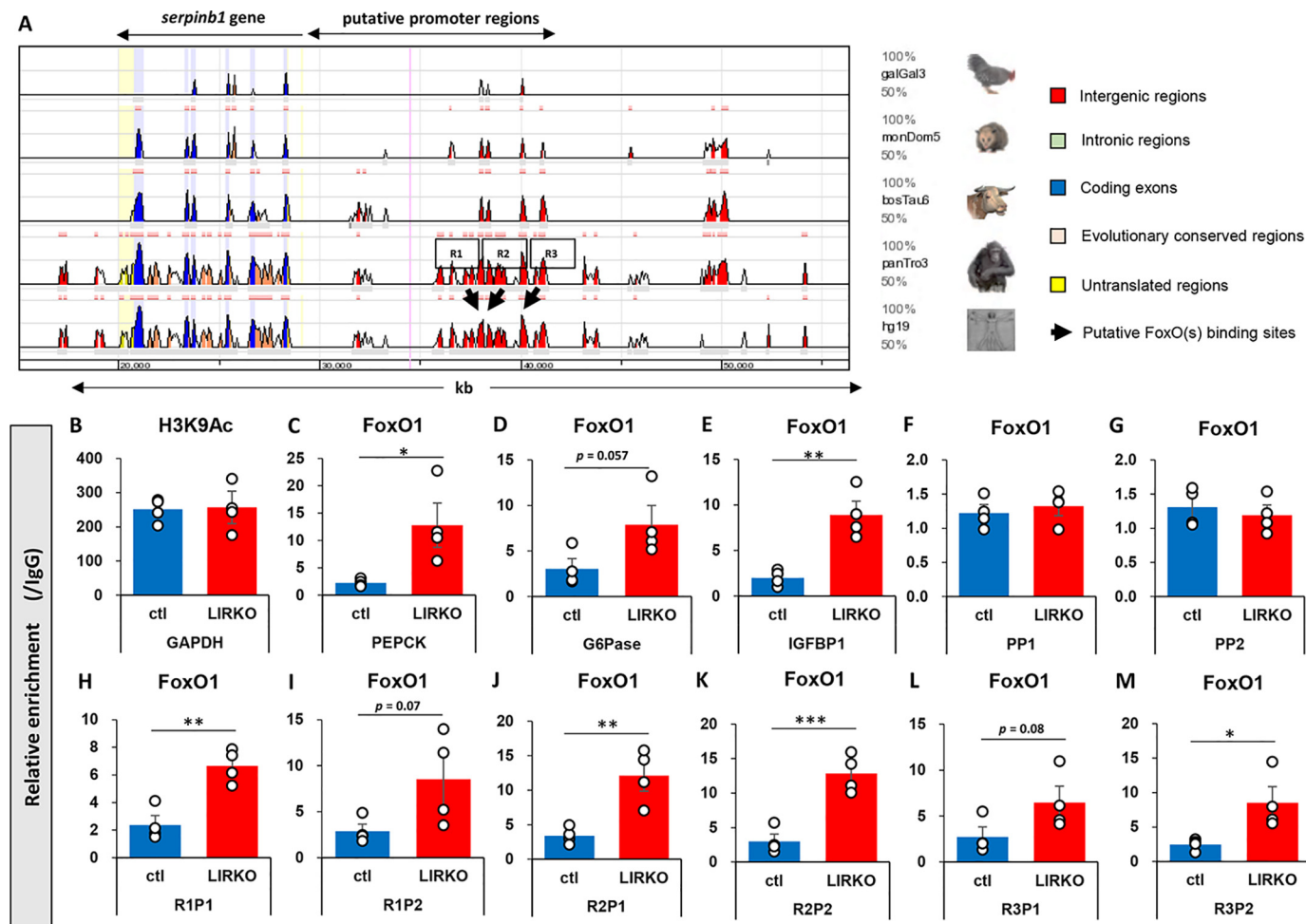
ting showed that the level of serpinB1 protein was markedly increased in the livers of LIRKO but not LIRFKO mice compared with floxed controls (Fig. 1, H and I).

#### Regulation of hepatic serpinB1 in FoxO transgenic and FoxO knockout mice

To better characterize the effects of FoxO1 on serpinB1 expression, we also examined hepatic expression of serpinB1 (and IGFBP-1) in WT mice and FoxO1 transgenic mice (Tgn), which express a constitutively active form of FoxO1 (TSS/A-FoxO1) in the liver (34). WT and Tgn mice were fasted for 18 h overnight and sacrificed 6 h after refeeding, when the function of endogenous FoxO proteins is suppressed but TSS/A-FoxO1 remains active in the liver of Tgn mice (34). As shown in Fig. 2A, liver IGFBP-1 expression was dramatically enhanced in refed Tgn mice compared with the WT, as reported previously (34). SerpinB1 mRNA levels were also markedly increased (~20-fold) in the liver of Tgn *versus* WT mice, and Western blotting confirmed that serpinB1 protein levels were substantially increased in livers from refed Tgn *versus* WT mice (Fig. 2, C and D). These results show that FoxO1 is sufficient to promote increased serpinB1 expression in the liver *in vivo*, similar to the effect of FoxO1 on IGFBP-1 expression.

In a related experiment, we also examined liver-specific FoxOKO mice in which the expression of FoxO1, FoxO3, and FoxO4 was selectively disrupted in the liver to determine whether endogenous FoxO proteins contribute to the expression of serpinB1 in the liver under conditions where the insulin receptor has not been deleted. Nine- to ten-week-old male FoxOKO and floxed littermate controls (FoxO<sup>fl/fl</sup>) were fasted

## FOXO1 regulates *serpinb1* in hepatocytes



**Figure 3. FoxO1 binds physically to sites upstream of the *serpinb1* gene.** *A*, alignment of *serpinb1* gene sequences from chicken, cow, mouse, and human. R1, R2, and R3 represent regions with high sequence homology. Arrows indicate putative FoxO-binding sites. *B*, ChIP assay for H3K9Ac on the *gapdh* promoter in primary hepatocytes from control (ctl) and LIRKO mice. *C–M*, ChIP assays for FoxO1 on *pepck*, *g6pase*, *igfbp-1*, or *serpinb1* genomic regions in primary hepatocytes from IR<sup>fl/fl</sup> and LIRKO mice. Data are mean  $\pm$  S.E. \*,  $p < 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$  ( $n = 4$ /group). The ChIP qPCR was performed with the primers described under “Experimental procedures.”

for 18 h prior to sacrifice, when insulin levels were low and endogenous FoxO proteins were active in FoxO<sup>fl/fl</sup> mice. Similar to IGFBP-1 (Fig. 2E), hepatic *serpinB1* mRNA levels were significantly reduced (by 70%) in 18-h-fasted FoxO<sup>fl/fl</sup> versus FoxO<sup>fl/fl</sup> mice (Fig. 2F). Hepatic *serpinB1* protein content, measured by Western blotting, also was lower in FoxO<sup>fl/fl</sup> versus FoxO<sup>fl/fl</sup> littermates (Fig. 2, G and H), demonstrating that endogenous FoxO proteins, including FoxO1 and perhaps other FoxO proteins, promote expression of *serpinB1* mRNA and protein in the liver.

### FoxO1 binds physically to sites upstream of *serpinb1*

Next, we sought to investigate whether FoxO1 binds directly to specific binding sites in the 5' upstream region of *serpinb1* (NC\_000079.6). Using the publicly available browser (<https://ecrbrowser.dcode.org>) (43),<sup>8</sup> we observed that *serpinb1* is located on mouse chromosome 13 between *serpinb1c* and *wrnip1* (GRCm38.p4 chr13: 32,842,092–32,851,185, complement). Alignment of genomic sequences across mouse, human,

and other species revealed that the 10-kb region upstream of *serpinb1* contains conserved intergenic binding sites for several transcription factors, including three highly conserved upstream regions (which we named R1, R2, and R3) located ~9 kb upstream from the *serpinB1* transcription initiation site. These sites share 75% sequence homology between mouse and human and contain putative FoxO-binding sites (Fig. 3A, arrows indicate localization of putative FoxO-binding sites). In particular, we identified two sites (R1 and R2) in upstream regions located at chr13: 32,859,879–32,860,181 in addition to a third site (R3) located at the chr13: 32,861,943–32,862,290 genomic region located ~9 kb upstream of the *serpinB1* transcription initiation site. In contrast, putative FoxO-binding sites were not identified in the proximal *serpinb1* promoter region.

Based on these data, we performed ChIP assays to test whether FoxO1 binds to these predicted sites in hepatocytes isolated from 4-month-old IR<sup>fl/fl</sup> and LIRKO mice. Following cross-linking, chromatin isolation and sonication, and precipitation with a specific antibody against FoxO1 or the IgG control, the enrichment of DNA fragments containing FoxO-binding sites was quantified by real-time PCR using two

<sup>8</sup> Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party-hosted site.

independent sets of primers that were designed for each of the three upstream regions containing a predicted FoxO-binding site or for the proximal *serpinb1* promoter, which did not contain a predicted high-affinity FoxO-binding site, as a control.

Preliminary studies of the ChIP assay were performed using an antibody against H3K9Ac, a posttranslationally modified form of histone 3 known to interact with the promoter region of *gapdh*. H3K9Ac was bound to the *gapdh* promoter after immunoprecipitation with anti-H3K9Ac antibody, and the relative enrichment was similar when the ChIP assay was conducted on control or LIRKO hepatocytes (Fig. 3B). We also confirmed that FoxO1 binding to the proximal promoter regions of several known FoxO1 target genes, including *pepck*, *g6pase*, and *igfbp-1*, which contain known FoxO-binding sites, was increased in hepatocytes from LIRKO mice compared with the floxed control (Fig. 3, C–E).

In contrast, we did not detect FoxO1 binding to unrelated sequences in the proximal promoter of *serpinb1*, as the relative chromatin enrichment containing this region of *serpinb1* promoter was similar after immunoprecipitation with anti-FoxO1 using two different primer sets (PP1 and PP2) in both control and LIRKO hepatocytes (Fig. 3, F and G). However, ChIP experiments demonstrated significant binding (2.4- to 2.9-fold) of FoxO1 to the predicted FoxO-binding sites in regions R1 (Fig. 3, H and I), R2 (Fig. 3, J and K), and R3 (Fig. 3, L and M) upstream of *serpinb1*, as assessed by two primer sets, and the recruitment of FoxO1 to these binding sites was increased significantly further in hepatocytes from LIRKO mice compared with the control by 2.3- to 3.9-fold. Together, these data indicate that FoxO1 binds directly to multiple conserved FoxO1-binding sites located upstream of *serpinb1* in hepatocytes and that FoxO1 binding to these sites is increased when insulin receptor signaling is disrupted in the liver.

#### Effects of insulin and FoxO1 on *serpinb1* gene expression in hepatocytes in vitro

We next asked whether insulin and/or FoxO1 regulate *serpinb1* gene expression in WT hepatocytes in a cell-autonomous manner. Mouse primary hepatocytes isolated from 4-month-old male C57BL6/J mice were stabilized in DMEM with 5 mM glucose and 0.1% fetal bovine serum (FBS) and then treated with DMEM with 5 mM glucose with or without 100 nM insulin for 1, 2, 4, 8, 12, or 24 h, followed by RNA extraction and measurement of *serpinB1* and IGFBP-1 transcripts. IGFBP-1 mRNA levels were reduced by 50% after 2-h incubation with insulin and continued to decline and remained suppressed throughout the 24-h incubation period (Fig. 4A), indicating that insulin effectively regulates the expression of IGFBP-1, a canonical FoxO1 target gene, in primary hepatocytes under these conditions. In contrast, insulin treatment had no significant effect on *serpinb1* gene expression at any time point in the study (Fig. 4B), suggesting that the effects of insulin on hepatocyte *serpinB1* expression may not be cell-autonomous and that other factors also are required.

To determine whether FoxO1 is sufficient to promote *serpinb1* gene expression in isolated hepatocytes, we transduced primary hepatocytes with adenovirus-expressing GFP alone

(Ad-GFP), GFP plus WT FoxO1 (Ad-FoxO1(WT)), or GFP plus a constitutively active form of FoxO1 (Ad-FoxO1(TSS/A)), and gene expression was assessed 48 h later for *serpinb1* and three other genes known to be regulated by FoxO1 (*igfbp-1*, *pepck*, and *g6pase*). At a multiplicity of infection (m.o.i.) of either 10 (Fig. 4, C–F) or 100 (Fig. 4, G–J) adenoviral particles per hepatocyte, transfection with Ad-FoxO1(WT) and Ad-FoxO1(TSS/A) increased the expression of IGFBP-1, PEPCK, and Glc-6-Pase compared with Ad-GFP, confirming that FoxO1 is sufficient to promote the expression of these target genes in hepatocytes in a cell-autonomous manner and that other factors required for transactivation of by FoxO1 (35) are present in these hepatocytes. In contrast, transfection with either Ad-FoxO1(WT) or Ad-FoxO1(TSS/A) failed to increase the expression of *serpinB1* (Fig. 4, C–J), suggesting that non-cell-autonomous factors may also be required for the regulation of *serpinB1* by FoxO proteins in the liver.

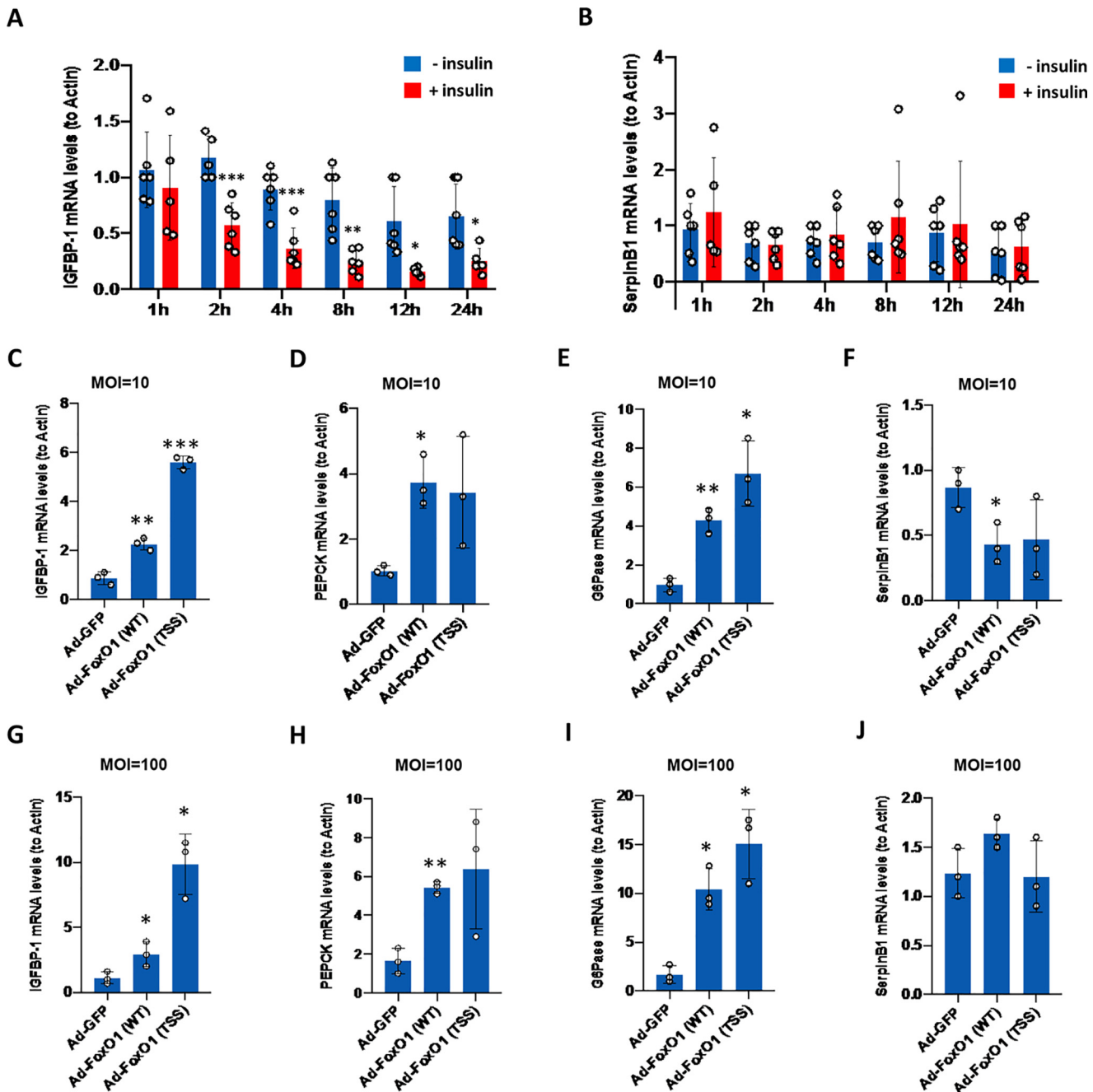
To test whether the lack of activation of *serpinB1* expression was due to an absence of binding of FoxO1 to the upstream binding sites in isolated hepatocytes, we also performed ChIP assays in primary hepatocytes infected with either Ad-GFP, Ad-FoxO1(WT), or Ad-FoxO1(TSS/A) and with an adenovirus expressing a DNA binding–defective form of FoxO1, Ad-FoxO1(H215R), which contains a point mutation within helix 3 of the DNA-binding domain that disrupts sequence-specific binding to the FoxO-binding site. As before, an antibody against H3K9Ac was used for the ChIP assay (Fig. 5A), and genes whose expression is controlled by FoxO1 and that contain known FoxO-binding sites within their proximal promoters (*pepck* or *g6pase*) were included as positive controls. As shown in Fig. 5, B and C, WT and TSS/A-FoxO1 are recruited to the PEPCK and Glc-6-Pase promoters whereas FoxO1 (H215R) is not, indicating that this binding is mediated through the FoxO1 DNA-binding domain. Similarly, adenoviral infection of either Ad-FoxO1(WT) or Ad-FoxO1(TSS/A) enhanced FoxO1-binding to the genomic DNA at distinct upstream sites of the *serpinB1* gene (Fig. 5, D–F). In contrast, binding was not enhanced by the expression of DNA binding–defective (H215R) FoxO1 (Fig. 5, D–F). These results indicate that FoxO1 is recruited to these sites through interactions with the FoxO1 DNA-binding domain. Taken together with gene expression studies (Fig. 4), these results also indicate that recruitment of FoxO1 to these sites is not sufficient to promote *serpinb1* gene expression in isolated hepatocytes.

Together, these data suggest the novel concept that, in contrast to *igfbp-1*, *pepck*, and *g6pase*, insulin treatment and FoxO1 are not sufficient to modulate *serpinb1* gene expression in isolated primary hepatocytes despite evidence supporting sequence-specific recruitment of FoxO1 to its binding sites upstream of the *serpinb1* gene and that non-cell-autonomous effects of FoxO1 may also contribute to the regulation of hepatic *serpinb1* expression by insulin *in vivo*.

#### Discussion

Diabetes mellitus is characterized by an absolute or relative scarcity of functional pancreatic islet insulin-secreting  $\beta$ -cells. Therapies that could increase pancreatic  $\beta$ -cell function or mass could potentially reverse the disease and halt its devastat-

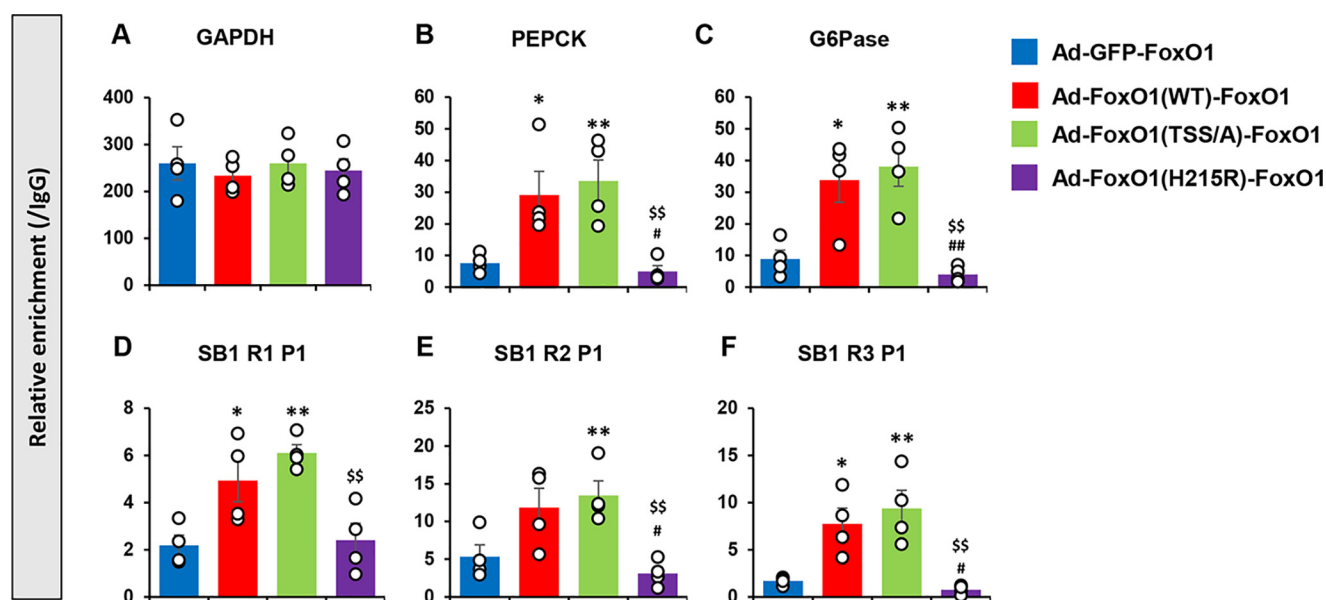
## FOXO1 regulates serpin1 in hepatocytes



**Figure 4. Effects of insulin and FoxO1 on serpinB1 gene expression in hepatocytes.** Primary hepatocytes isolated from 4-month-old male C57BL6/J mice were stabilized in DMEM containing 5 mM glucose and 0.1% FBS overnight prior to treatment with (*red columns*) or without (*blue columns*) 100 nM of insulin in DMEM and 5 mM glucose for 1, 2, 4, 8, 12, or 24 h; RNAs were extracted and then *serpinB1* transcript levels were assessed by qRT-PCR. *A*, relative quantification of hepatocyte *igfbp-1* mRNA levels in the presence or absence of insulin. Data represent mean  $\pm$  S.E. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$  ( $n = 6$ /group). *B*, relative quantification of liver *serpinB1* mRNA in the presence (*red columns*) or absence (*blue columns*) of insulin. *C–F*, relative quantification by qRT-PCR (normalized to actin) of liver *igfbp-1* (*C*), *pepck* (*D*), *g6pase* (*E*), and *serpinB1* (*F*) in mouse primary hepatocytes 40 h after transfection at an m.o.i. of 10, with adenoviruses expressing GFP (*Ad-GFP*), GFP plus WT FoxO1 (*Ad-FoxO1(WT)*), or GFP plus constitutively active FoxO1 (*Ad-FoxO1(TSS/A)*). Data represent mean  $\pm$  S.E. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$  ( $n = 3$ /group). *G–J*, relative quantification by qRT-PCR (normalized to actin) of liver *igfbp-1* (*G*), *pepck* (*H*), *g6pase* (*I*), and *serpinB1* (*J*) in hepatocytes infected for 48 h at an m.o.i. of 100. Data represent mean  $\pm$  S.E. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$  ( $n = 3$ /group).

ing complications (36). The last decade has witnessed a substantial effort in devising methods to generate pancreatic  $\beta$ -cells (4–12, 15–17). Previous studies have shown that compensatory  $\beta$ -cell proliferation is a common response to insulin resistance in a variety of settings, including obesity (21). Islet mass and  $\beta$ -cell proliferation are markedly increased in LIRKO mice, and we have shown that circulating endogenous factors produced by the liver (hepatokines) contribute to this effect,

including serpinB1, which we found to have pancreatic  $\beta$ -cell proliferation-promoting activity in multiple species (6, 17). Although hepatic expression and circulating levels of serpinB1 are markedly elevated in LIRKO mice, little is known regarding specific mechanisms involved in regulating the expression of serpinB1 in the liver. Here we show that FoxO1 plays an important role in promoting serpinB1 expression *in vivo* in the setting of hepatic insulin resistance.



**Figure 5. FoxO1 binds to sites upstream of the *serpinb1* gene in isolated hepatocytes.** ChIP assay was performed in mouse primary hepatocytes 40 h after transfection at a multiplicity of infection of 30 with an adenovirus expressing GFP (*Ad-GFP*), GFP plus WT FoxO1 (*Ad-FoxO1(WT)*), GFP plus constitutively active FoxO1 (*Ad-FoxO1(TSS/A)*) or GFP plus a DNA binding–defective FoxO1 (*Ad-FoxO1(H215R)*). *A*, ChIP assay for H3K9Ac on the *gapdh* promoter. *B* and *C*, ChIP assays for FoxO1 on *pepck* or *g6pase* genomic regions in primary murine hepatocytes. *D–F*, ChIP assay for FoxO1 on genomic upstream regions of *serpinb1* (R1, R2, and R3). Data are mean  $\pm$  S.E. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus GFP control; #,  $p < 0.05$ ; ##,  $p < 0.01$  versus WT FoxO1; \$\$,  $p < 0.01$  versus TSS-A FoxO1 ( $n = 4$ /group). The ChIP qPCR was performed with the primers described under “Experimental procedures.”

Previously, we reported that knocking out FoxO1 in the liver is sufficient to restore the ability of insulin to maintain glucose homeostasis and prevent  $\beta$ -cell hyperplasia in LIRKO mice (25). Here we show that the effect of knocking out FoxO1 in the liver on  $\beta$ -cell mass reflects changes in  $\beta$ -cell proliferation and the expression of serpinB1, indicating that effects of hepatic insulin resistance on serpinB1 expression and  $\beta$ -cell proliferation are FoxO1-dependent. Further, studies in transgenic mice expressing a constitutively active form of FoxO1 in the liver demonstrate that FoxO1 is also sufficient to enhance hepatic serpinB1 expression in mice in which insulin signaling has not been disrupted in the liver. Further, studies in liver-specific FoxO knockout mice show that endogenous FoxO proteins (including FoxO1, FoxO3, and FoxO4) contribute to hepatic expression of serpinB1 under fasting conditions, when insulin levels and effects are suppressed. Taken together, these results demonstrate that FoxO proteins promote the expression of serpinB1 in the liver and support the concept that FoxO-dependent effects on the expression of serpinB1 and possibly other factors may contribute to increased  $\beta$ -cell proliferation and mass in the setting of hepatic insulin resistance.

We also found that the regulation of serpinB1 by FoxO1 and insulin differs from that of several known FoxO target genes in the liver. Previous studies have emphasized cell-autonomous effects of FoxO proteins on gene expression because of recruitment to *cis*-acting elements through sequence-specific interactions with FoxO-binding sites often located within the proximal promoters of target genes. In this study, computer analysis identified several FoxO1-binding sites located  $\sim 9$  kb upstream of the *serpinb1* gene, and ChIP studies confirmed that FoxO1 is recruited to these upstream sites. In contrast, no putative FoxO-binding sites were identified in the *serpinb1* proximal promoter, and ChIP studies showed that FoxO1 is also not

recruited to the proximal promoter region of the *serpinb1* gene through other mechanisms, *e.g.* protein–protein interactions. Adenoviral expression of WT and constitutively active FoxO1 was sufficient to enhance, and insulin treatment was sufficient to suppress, the expression of IGFBP-1, PEPCK, and Glc-6-Pase, consistent with previous studies, but not serpinB1, in isolated hepatocytes. These findings suggest that other factors also are required for FoxO proteins to effectively promote the expression of serpinB1 and suggest the novel concept that FoxO1 and insulin regulate serpinB1 expression in the liver in a non-cell-autonomous fashion.

It is interesting to note that these differences in the regulation of serpinB1 versus IGFBP-1, PEPCK, and Glc-6-Pase correspond to differences in the organization of FoxO-binding sites in the 5' upstream regions of the *serpinb1*, *igfbp-1*, *pepck*, and *g6pase* genes. Functional FoxO-binding sites are most often located in the proximal promoter region and are found within several hundred base pairs of the transcription initiation site of the *igfbp-1*, *pepck*, and *g6pase* genes, where FoxO1 is able to bind and function together with other and *trans*-acting factors to stimulate gene expression directly. In this study, ChIP assays confirmed that FoxO1 interacts with the proximal promoter region of the *igfbp-1*, *pepck*, and *g6pase* genes and that this interaction is increased when the insulin receptor is knocked out in hepatocytes, consistent with previous studies indicating that FoxO1 directly contributes to insulin regulation of these genes. In contrast, ChIP studies showed that FoxO1 does not interact with elements located within the proximal *serpinb1* promoter but, instead, that FoxO1 is recruited to several conserved FoxO-binding sites located further upstream of the *serpinb1* gene, and this binding is also enhanced when the insulin receptor is knocked out. Furthermore, the binding of FoxO1 to this upstream region increases when the con-

## FOXO1 regulates serpin1 in hepatocytes

stitutively active form of FoxO1 (TSS/A) is expressed but not the DNA binding–defective FoxO1 (H215R) mutant. Taken together, these results indicate that the recruitment of FoxO1 to these upstream binding sites involves sequence-specific interaction with FoxO-binding sites but that is not sufficient to enhance the expression of *serpin1* in isolated hepatocytes and that other factors are also required for driving the transcription of the *serpin1* gene in the liver when insulin signaling is impaired.

Recent studies indicate that the extrahepatic effects of FoxO1 are also important in mediating other effects of hepatic insulin resistance, including the ability of insulin to regulate hepatic glucose production (HGP). Studies in LIRKO and LIRFKO mice indicate that insulin regulates HGP in a FoxO1-dependent fashion (25, 37), and yet insulin and FoxO1 have limited effects on glucose production in isolated hepatocytes (37). This apparent discrepancy has been resolved by the recognition that the ability of insulin to suppress lipolysis in white adipose tissue (WAT) and the flux of nonesterified fatty acids to the liver, where fatty acid oxidation supports and promotes gluconeogenesis, is critical for the ability of insulin to suppress HGP (38). Titchenell et al. (32) reported that the ability of insulin to suppress WAT lipolysis is impaired when insulin signaling is disrupted in the liver and that knocking out FoxO1 in the liver restores the ability of insulin to suppress WAT lipolysis and, thereby, regulate HGP. Thus, in addition to its direct, cell-autonomous effects on the expression of gluconeogenic and glycolytic genes in the liver, hepatic FoxO1 also impacts the ability of insulin to regulate HGP through indirect, non-cell-autonomous mechanisms. Based on our results, it is interesting to speculate that FoxO1-dependent mechanisms promoting the expression of liver serpinB1 may also involve non-cell-autonomous effects and communication with factors derived from other cells or tissues in the setting of hepatic insulin resistance. We observed that other genes, including the soluble leptin receptor and growth hormone receptor, are also regulated by FoxO1 in the liver *in vivo* but do not respond to transfection with FoxO1 expression vectors in isolated hepatocytes.<sup>9</sup> Together, these observations support the novel concept that FoxO1 contributes to the regulation of hepatic gene expression and metabolism through both cell-autonomous and non-autonomous mechanisms.

In summary, we found that FoxO1-dependent mechanisms promote the expression of serpinB1 when insulin signaling is disrupted in the liver, concomitant with effects on  $\beta$ -cell proliferation. FoxO proteins are required and sufficient to promote serpinB1 gene expression *in vivo* and interact with FoxO binding sites in multiple conserved binding sites upstream of the *serpin1* gene. However, unlike other FoxO target genes, neither insulin nor FoxO1 is sufficient to regulate the expression of serpinB1 in primary hepatocytes, suggesting that non-cell-autonomous factors are also involved in mediating the effects of insulin and FoxO1 on the expression of serpinB1 in the liver. These results support the broader concept that FoxO proteins function in the context of a complex regulatory network that

includes both cell-autonomous and non-autonomous mechanisms involved in the control of metabolism. A potential role of other regulatory pathways and transcription factors contributing to mediating the expression of serpinB1 cannot be ruled out and requires further study. A better understanding of the mechanism(s) mediating the effects of insulin and FoxO proteins and potentially other transcription factors on the expression of serpinB1 may provide additional insight into mechanisms contributing to the regulation of gene expression and metabolism in hepatic insulin resistance and provide opportunities to selectively target hepatic serpinB1 expression to increase the number of functional  $\beta$ -cells in patients with diabetes.

## Experimental procedures

### Animals

Mouse studies were performed at the Jesse Brown Veterans Affairs Medical Center after approval by its institutional animal care committee. Male liver-specific transgenic mice expressing a modified form of human FoxO1, in which all three Akt phosphorylation sites (Thr-24, Ser-256, and Ser-319) were replaced with alanine residues (TSS/A-FoxO1), were crossed with female FVB/N WT female mice (Harlan Laboratories) to generate transgenic and WT littermate controls for studies (34). Liver-specific FoxOKO mice, in which the alleles for FoxO1, FoxO3, and FoxO4 are disrupted in the liver, were generated by crossing FoxO<sup>fl/fl</sup> mice (from Dr. Ron DePinho) with albumin-Cre (The Jackson Laboratory) mice (39). LIRKO and LIRFKO mice were generated previously by crossing albumin-Cre (The Jackson Laboratory) with IR<sup>fl/fl</sup> mice (provided by C. R. Kahn, Harvard Medical School) and FoxO1<sup>fl/fl</sup> mice (provided by Ronald DePinho, MD Anderson Cancer Center), as described previously (25). Mice were housed on a 12-h:12-h light:dark cycle with lights off at 18:00 and provided standard chow. Plasma insulin was detected by ELISA (Crystal Chem).

### Fasting/feeding studies

To evaluate the ability of FoxO1 to promote the expression of SerpinB1 in the liver, 10-week-old male FoxO1 transgenic mice and WT littermates were fasted for 18 h overnight, refed at 9 a.m., and sacrificed 6 h after refeeding, when the function of endogenous FoxO proteins is suppressed in the liver and the transgenic TSS/A-FoxO1 remains active, similar to previous studies (34, 39). Conversely, to evaluate the role of endogenous FoxO proteins in promoting the expression of serpinB1 in the liver, liver-specific FoxO KO mice and floxed littermates were sacrificed at the end of an 18-h overnight fast, when insulin levels are low and endogenous FoxO proteins are active in the liver, as before (39).

### Islet studies

For studies of  $\beta$ -cell proliferation, 10-week-old freely fed male LIRKO, LIRFKO, IR<sup>fl/fl</sup>, and IR<sup>fl/fl</sup>FoxO1<sup>fl/fl</sup> mice were sacrificed between 10 and 11 a.m. by decapitation following brief sedation with isoflurane. Following collection of cervical blood, the liver was freeze-clamped with aluminum tongs precooled with liquid N<sub>2</sub> for storage at  $-80^{\circ}\text{C}$ , and the pancreas was fixed

<sup>9</sup>T. Unterman and W. Zhang, unpublished observations.



overnight in Z-Fix (Anatech) and embedded in paraffin prior to sectioning in the University of Illinois Histology Core as before (25). For analysis of islet mass, tissue sections (4  $\mu\text{m}$  thick) were probed with anti-insulin antibody (Abcam) and then scanned at  $\times 200$  with an Aperio Scanscope CS for quantification of total tissue and islet cross-sectional area using ImageScope software (Aperio). For analysis of  $\beta$ -cell proliferation, sections were immunostained with anti-Ki67 (BD Biosciences) and anti-insulin (Abcam) antibodies. Cell counting was performed manually in a blinded fashion by a single observer. Insulin<sup>+</sup> and Ki67<sup>+</sup>  $\beta$ -cells were identified using a fluorescence microscope (Olympus). Insulin<sup>+</sup> cells showing nuclear 4',6-diamidino-2-phenylindole staining were considered  $\beta$ -cells. Insulin<sup>+</sup> cells showing nuclear colocalized staining for 4',6-diamidino-2-phenylindole<sup>+</sup> and Ki67<sup>+</sup> were counted as proliferating  $\beta$ -cells as described previously (6, 17), and at least 1200  $\beta$ -cells per pancreas were counted. At sacrifice, mice were briefly sedated with isoflurane prior to decapitation. The liver was snap-frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  until analysis.

### Hepatocytes and adenoviral vectors

As we described previously (17), hepatocytes were isolated from male mice by collagenase digestion via portal vein perfusion. Hepatocytes were washed twice in hepatocyte wash medium (Invitrogen) and then seeded in collagen-coated 6-well plates (BD BioCoat) at a density of  $10^6$  cells/well in DMEM containing 25 mM glucose and 10% FBS (v/v) for 4–6 h before further treatment. For studies of hepatocytes from LIRKO and IR<sup>fl/fl</sup> mice, plated cells were rinsed with PBS and refed with DMEM, 25 mM glucose, and 10% FBS and harvested 24 h later for ChIP analysis. For studies of insulin effects on gene expression, plated hepatocytes from WT C57Bl/6 mice were rinsed with PBS and then refed with DMEM plus 5 mM glucose and 0.1% FBS for 16 h prior to treatment with DMEM containing 5 mM glucose with or without 100 nM insulin for 1–24 h. For studies of FoxO with adenoviral vectors, plated hepatocytes from WT C57Bl/6 were rinsed with PBS and refed with DMEM, 25 mM glucose, 10% FBS and transfected with adenoviral vectors (10–100 infectious units/cell) for 16 h when cells were refed with DMEM, 25 mM glucose, and 10% FBS. Hepatocytes were harvested 24 h later for analysis of gene expression or ChIP studies. Adenoviral vectors, which express GFP alone or GFP plus WT FoxO1, TSS-A FoxO1, or H215R FoxO1 were described previously (34, 40).

### ChIP assays

Hepatocytes were fixed in 1% formaldehyde and then quenched with glycine. Cells were washed with PBS and lysed in cold lysis buffer with protease and phosphatase inhibitors (Sigma). Lysates were then sonicated (Branson Digital Sonifier 450) using 25% power and 10-s on/off pulses for a total of 3 min, yielding chromatin fragments of 100 to 1000 bp. Immunoprecipitations were performed using anti-FoxO1 antibody (Abcam, ab39670), anti-acetyl-histone H3 (Lys-14) (Cell Signaling Technology, 5275), anti-acetyl-histone H3 (Lys-9) (Cell Signaling Technology, 9649), or rabbit IgG (Cell Signaling Technology, 2729), and protein/cross-linking was reversed.

Normalization of relative enrichment of FoxO1 on promoter regions was performed for both input chromatin and control IgG background levels. The relative enrichment of DNA binding was measured by qPCR using primers for IGFBP-1 (5'-ATC-TGGCTAGCAGCTTGCTGA-3' (forward) and 5'-CCGTGTG-CAGTGTTCAATGCT-3' (reverse)), Glc-6-Pase (5'-GCCTCT-AGCACTCAAGCAGTG-3' (forward) and 5'-TGTGCCCTGC-CCCTGTTTTATATG-3' (reverse)), PEPCK (5'-TCCACCACA-CACCTAGTGAGG-3' (forward) and 5'-AGGGCAGGCCTAG-CCGAGACG-3' (reverse)), SerpinB1 R1P1 (5'-ACCTCCTCAA-GACCTGTGGA-3' (forward) and 5'-GCTGGAGATGCCTTA-CCAGG-3' (reverse)), SerpinB1 R1P2 (5'-GCCAGTTACCTCC-TCAAGACC-3' (forward) and 5'-CCAGGGTGGAAAGCTT-AGCA-3' (reverse)), SerpinB1 R2P1 (5'-TGCTAAGCTTTCC-ACCCTGG-3' (forward) and 5'-CCCTCTACCCCAAGACC-TCA-3' (reverse)), SerpinB1 R2P2 (5'-CCTGGTAAGGCATC-TCCAGC-3' (forward) and 5'-TACCACTGAGCCCTCT-ACCC-3' (reverse)), SerpinB1 R3P1 (5'-CTCAGCCTCTCAC-CTTCTGC-3' (forward) and 5'-CTGACTTGGCCCTTGTG-GAT-3' (reverse)), SerpinB1 R3P2 (5'-TCGCCATGGGACCA-AAAGAA-3' (forward) and 5'-GTTCCCTTCTGGAGCT-CAC-3' (reverse)), SerpinB1 PP1 (5'-TCCTCTGAAGAAAG-ACAAGAAGCA-3' (forward), 5'-CAATGGCCTGGGCTAG-TCAA-3' (reverse)), and SerpinB1 PP2 (5'-AGGAGAATAAA-GCTGAGCCCA-3' (forward) and 5'-AATAGTGGGGAATT-GTGGGTTGA-3' (reverse)).

### RT-PCR

For real-time PCR experiments, total RNAs were extracted from the liver or hepatocytes using the TRIzol method (41). 1  $\mu\text{g}$  of total RNA was used for a reverse transcription reaction using the high-capacity cDNA Archive Kit (Applied Biosystems). cDNA was analyzed using the ABI 7900HT system (Applied Biosystems). The level of TATA-binding protein (TBP) mRNA transcripts was used as an internal control. The following primers were used: SerpinB1 (5'-GCTGCTACAGG-AGGCATTGC-3' (forward) and 5'-CGGATGGTCCACTGT-GAATTC-3' (reverse)), PEPCK (5'-TGTTTACTGGGAA-GGCATCG-3' (forward) and 5'-AGGTCTACGGCCACCA-AAG-3' (reverse)), Glc-6-Pase (5'-ATCCGGGGCATCTACA-ATG-3' (forward) and 5'-TGGCAAAGGGTGTAGTGTCA-3' (reverse)), IGFBP-1 (5'-GGAGATCGCCGACCTCAAG-3' (forward) and 5'-CTGCAGCTAATCTCTCTAGCACTTTAT-3' (reverse)), and TATA-binding protein (5'-ACCCTTACCAAT-GACTCCTATG-3' (forward) and 5'-ATGATGACTGCAGCA-AATCGC-3' (reverse)).

### Western blotting

Tissue samples were lysed in M-PER buffer (Thermo Fisher Scientific), and total protein concentration was measured by BCA assay (Pierce). Samples were resuspended in Laemmli buffer with  $\beta$ -mercaptoethanol, boiled, and resolved by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes, blocked in PBS containing 5% BSA and 0.1% Tween 20, and incubated with rabbit antiserum to human SerpinB1 (42), GAPDH (Cell Signaling Technology, 5174P), or mouse antiserum to actin (Santa Cruz Biotechnology, sc-1615) or  $\beta$ -tubulin (Santa Cruz Biotechnology, sc-5274). Secondary goat anti-rab-

## FOXO1 regulates serpinb1 in hepatocytes

bit (Santa Cruz Biotechnology, sc-2054) or goat anti-mouse (Santa Cruz Biotechnology, sc-2055) was used thereafter. Protein bands were quantified using ImageJ software.

### Statistical analysis

All data are presented as mean  $\pm$  S.E. Data were analyzed using unpaired, two-tailed Student's *t* test.  $p < 0.05$  was considered statistically significant.

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