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

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Running title: FOXO1 regulates *serpinb1* in hepatocytes

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Abstract

FoxO proteins are major targets of insulin action and FoxO1 mediates effects of insulin on hepatic glucose metabolism. We previously reported that serpinB1 is a liver secreted factor (hepatokine) that promotes adaptive β-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 cell non-autonomous manner. Mice lacking both insulin receptor and FoxO1 (LIRFKO) exhibit reduced β-cell mass compared to LIRKO mice, due to attenuation of β-cell proliferation. While hepatic expression of serpinB1 mRNA and protein levels were increased in LIRKO mice, both 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. Chromatin immunoprecipitation studies demonstrated that FoxO1 binds to 3 distinct sites located ~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 wild type or constitutively active FoxO1, and insulin treatment is sufficient to regulate other FoxO1 target genes (IGFBP-1, PEPCK), but not serpinB1 expression in mouse primary hepatocytes. These results indicate that liver FoxO1 promotes serpinB1 expression in hepatic insulin resistance, and that cell non-autonomous factors contribute to FoxO1-dependent effects on serpinB1 expression in the liver.

Introduction

Diabetes mellitus develops when the number or function of pancreatic β-cells is insufficient to maintain normoglycemia. Typically, the mass of insulin-producing β-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 β-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 (9,10,16) or other sources (4,8,11,13,16) and the proliferation of pre-existing islet β-cells (5-7,12,17). The evolving stem cell-based approaches coupled with their oncogenic potential after transplantation suggests it is not yet ready for therapeutic consideration (18). Current strategies have focused on harnessing the replicative activity (19,20) of β-cells that is known to increase significantly in response to challenges such as insulin resistance (19,21-23). Thus the identification of endogenous molecules that selectively enhance human β-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 β-cell growth factor (6). While 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 resistance, including liver-specific insulin receptor knockout (LIRKO) mice, a unique model of islet β-cell hyperplasia (17,22). SerpinB1 also stimulates islet β-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 (26-28). Insulin activates Akt which induces phosphorylation at 3 highly conserved sites corresponding to Thr24, Ser256 and Ser319 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 in liver-specific IR knockout (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 due to 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 β-cell hyperplasia in LIRKO mice (25). Since serpinB1 plays an important role in mediating effects of hepatic insulin resistance on β-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 cell non-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

β-cell proliferation and hepatic serpinB1 expression in LIRKO *vs.* LIRFKO mice: We first asked whether hepatic FoxO1 plays a critical role in promoting islet β-cell proliferation in the setting of hepatic insulin resistance using liver-specific insulin receptor knockout (LIRKO) and IR/FoxO1 double knockout (LIRFKO) mice. We measured islet mass and β-cell replication in 10-week-old male insulin receptor floxed ($IR^{fl/fl}$), LIRKO and LIRFKO mice by insulin and Ki67 co-immunostaining. As shown in Figs. 1A-1D, islet area, islet mass and β-cell proliferation were increased 3-to-4 fold in LIRKO compared to $IR^{fl/fl}$ littermate controls consistent with previous studies (6,17,22). In contrast, islet mass and β-cell proliferation were not increased in LIRFKO mice compared to floxed controls (Figs. 1A-1D), indicating that FoxO1 plays an important role in promoting β-cell proliferation in the setting of hepatic insulin resistance. In agreement with changes in islet β-cell proliferation and area, LIRKO mice exhibited 11-fold higher insulin levels compared to control mice and this effect, also was largely reversed in LIRFKO mice (Fig. 1E).

Next, we assessed the abundance of serpinB1 messenger RNA (mRNA) and protein levels in the liver of LIRKO, LIRFKO and $IR^{fl/fl}$ mice. For comparison, we also assessed the expression of insulin-like growth factor binding protein 1 (IGFBP-1), a canonical FoxO1 target gene which 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 as compared to IR floxed ($IR^{fl/fl}$) or IR/FoxO1 floxed ($IR^{fl/fl}F^{fl/fl}$) mice, while IGFBP-1 mRNA levels were not increased in LIRFKO-derived livers relative to $IR^{fl/fl}$ and $IR^{fl/fl}F^{fl/fl}$ mice (Fig. 1F). Gene expression studies showed that liver serpinB1 mRNA levels also are increased ~70-fold in LIRKO compared to $IR^{fl/fl}$ mice (Fig. 1G), consistent with previous studies (6). In contrast, LIRFKO mice displayed no increase in serpinB1 gene expression compared to 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, though effects on pre-mRNA or mRNA stability cannot be ruled out. Similarly, western blotting showed that the level of serpinB1 protein was markedly increased in the livers of LIRKO, but not LIRFKO mice, compared to floxed controls (Figs. 1H and 1I).

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 18 hr overnight and sacrificed 6 hr 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 to WT, as previously reported (34). SerpinB1 mRNA levels also were markedly increased (~20-fold) in the liver of Tgn *vs.* WT mice and western blotting confirmed that serpinB1 protein levels were substantially increased in liver from refed Tgn *vs.* WT mice (Figs. 2C and 2D). 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 ($FoxO^{fl/fl}$) were fasted for 18 hours prior to sacrifice, when insulin levels are low and endogenous FoxO proteins are active in $FoxO^{fl/fl}$ mice. Similar to IGFBP-1 (Fig. 2E), hepatic serpinB1 mRNA levels were significantly reduced (by 70%) in 18-hr fasted FoxOKO *vs.* $FoxO^{fl/fl}$ mice (Fig. 2F). Hepatic serpinB1 protein content measured by western blotting also was lower in FoxOKO *vs.* $FoxO^{fl/fl}$ littermates (Figs. 2G and 2H), 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 from *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, 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-kilobase (kb) region upstream from *serpinb1* contains conserved intergenic binding sites for several transcription factors, including 3 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 chr13: 32,861,943 – 32,862,290 genomic region located ~9 kb upstream from the serpinB1 transcription initiation site. In contrast, putative FoxO binding sites were not identified in the proximal *serpinb1* promoter region.

Based on this data, we performed chromatin immunoprecipitation (ChIP) assays to test whether FoxO1 binds to these predicted sites in hepatocytes isolated from 4-month-old $IR^{fl/fl}$ and LIRKO mice. Following crosslinking, chromatin isolation and sonication, and precipitation with a specific antibody against FoxO1 or IgG control, the enrichment of DNA fragments containing FoxO binding sites was quantified by real-time PCR using two independent sets of primers that were designed for each of the 3 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 on the ChIP assay were performed using an antibody against H3K9Ac, a post-translationally modified form of the 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 phosphoenolpyruvate carboxykinase (*pepck)*, glucose-6 phosphatase (*g6pase)* and *igfbp-1,* which contain known FoxO binding sites, was

increased in hepatocytes from LIRKO mice compared to floxed control (Fig. 3C-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 (Figs. 3F and 3G). However, ChIP experiments demonstrated significant binding (2.4 to 2.9-fold) of FoxO1 to the predicted FoxO binding sites in regions R1 (Fig. 3H-I), R2 (Fig. 3J-K) and R3 (Fig. 3L-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 to 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 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 wild type hepatocytes in a cell autonomous manner. Mouse primary hepatocytes isolated from 4-month-old male C57BL6/J mice were stabilized in DMEM with 5mM glucose and 0.1% fetal bovine serum (FBS) and then treated with DMEM with 5mM glucose with/without 100 nM insulin for 1, 2, 4, 8, 12 or 24 hours followed by RNA extraction and measurement of serpinB1 and IGFBP-1 transcripts. IGFBP-1 mRNA levels were reduced by 50% after a 2h-incubation with insulin, and continued to decline and remained suppressed throughout the 24h-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 upon *serpinb1* gene expression at any time point in the study (Fig. 4B), suggesting that 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 green fluorescent protein (GFP) alone (Ad-GFP), GFP plus wild type FoxO1 (Ad-FoxO1(WT)) or GFP plus a constitutively active form of FoxO1 (Ad-FoxO1(TSS/A)) and gene expression was assessed 48 hr later for *serpinb1* and 3 other genes known to be regulated by FoxO1 (*igfbp-1*, *pepck* and *g6pase*). At a multiplicity of infection (MOI) of either 10 (Fig. 4C-4F) or 100 (Fig. 4D-4J) adenoviral particles per hepatocyte, transfection with Ad-FoxO1(WT) and Ad-FoxO1(TSS/A) increased the expression of IGFBP-1, PEPCK and G6Pase when compared to 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 (Figs. 4C-J), suggesting that cell non-autonomous factors also may 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), 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 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 contain known FoxO binding sites within their proximal promoters (*pepck* or *g6pase*) were included as positive controls. As shown in Fig. 5B-C, WT and TSSS/A-FoxO1 are recruited to the PEPCK and G6Pase promoters, while 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 the FoxO1 binding to the genomic DNA at distinct upstream sites of the serpinB1 gene (Fig. 5D-F). In contrast, binding was not enhanced by the expression DNA-binding defective (H215R) FoxO1 (Fig. 5D-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 the evidence supporting sequence-specific recruitment of FoxO1 to its binding sites upstream from the *serpinb1* gene, and that cell non-autonomous effects of FoxO1 also may 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 β-cells. Therapies that could increase pancreatic β-cell function or mass could potentially reverse the disease and halt its devastating complications (36). The last decade has witnessed a substantial effort in devising methods to generate pancreatic β-cells (4-12,15-17). Previous studies have shown that compensatory β-cell proliferation is a common response to insulin resistance in a variety of settings including obesity (21). Islet mass and β-cell proliferation are markedly increased in liver-specific insulin receptor knockout (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 β-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.

Previously, we reported that knocking out FoxO1 in the liver is sufficient to restore the ability of insulin to maintain glucose homeostasis and prevent β-cell hyperplasia in LIRKO mice (25). Here, we show that the effect of knocking out FoxO1 in the liver on β-cell mass reflects changes in β-cell proliferation and the expression of serpinB1, indicating that effects of hepatic insulin resistance on serpinB1 expression and β-cell proliferation are FoxO1-dependent. Further, studies in transgenic mice expressing a constitutively active form of FoxO1 in the liver demonstrate that FoxO1 also is sufficient to enhance hepatic serpinB1 expression in mice where 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 β-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 due to 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 ~9 kp upstream from 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 also is not recruited to the proximal

promoter region of *serpinb1* gene through other mechanisms, e.g., protein-protein interactions. Adenoviral expression of wild type and constitutively active FoxO1 was sufficient to enhance, and insulin treatment was sufficient to suppress the expression of IGFBP-1, PEPCK and G6Pase, 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 cell non-autonomous fashion.

It is interesting to note that these differences in the regulation of serpinB1 *vs.* IGFBP-1, PEPCK and G6Pase 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 FoxO1 is recruited to several conserved FoxO binding sites located further upstream from the *serpinb1* gene, and this binding also is enhanced when the insulin receptor is knocked out. Furthermore, the binding of FoxO1 to this upstream region increases when the constitutively 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 is not sufficient to enhance the expression of *serpinb1* in isolated hepatocytes, and that other factors are also required for driving the transcription of the *serpinb1* gene in the liver when insulin signaling is impaired.

Recent studies indicate that extrahepatic effects of FoxO1 also are 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, 38), and yet insulin and FoxO1 have limited effects on glucose production in isolated hepatocytes (38). 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 non-esterified fatty acids to liver where fatty acid oxidation supports and promotes gluconeogenesis, is critical for the ability of insulin to suppress HGP (39). Titchenell and colleagues 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 (32). 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, cell non-autonomous mechanisms. Based on our results, it is interesting to speculate that FoxO1-dependent mechanisms promoting the expression of liver serpinB1 also may involve cell non-autonomous effects and communication with factors derived from other cells or tissues in the setting of hepatic insulin resistance. We have observed that other genes, including the soluble leptin receptor and growth hormone receptor, also are regulated by FoxO1 in the liver *in vivo* but do not respond to transfection with FoxO1 expression vectors in isolated hepatocytes (T. Unterman and W. Zhang, unpublished observations). 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 have found that FoxO1-dependent mechanisms promote the expression

serpinB1 when insulin signaling is disrupted in the liver concomitant with effects on β-cell proliferation. FoxO proteins are required and sufficient to promote serpinB1 gene expression *in vivo*, and interact with FoxO binding sites in multiple conserved upstream binding sites from the *serpinb1* gene. However, unlike other FoxO target genes, neither insulin nor FoxO1 is sufficient to regulate the expression of serpinB1 in primary hepatocytes, suggesting that cell non-autonomous factors also are involved in mediating effects of insulin and FoxO1 on the expression of serpinB1 in the liver. These results support the broader concept that FoxO proteins function within 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 for other regulatory pathways and transcription factors contributing to mediating the expression of serpinB1 cannot be ruled out and requires further study. Better understanding of the mechanism(s) mediating 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 β-cells in patients with diabetes.

Experimental Procedures

Animals–Mouse studies were performed at the Jesse Brown VA Medical Center after approval by the JBVAMC institutional animal care committee. Male liver-specific transgenic mice expressing a modified form of human FoxO1, in which all three Akt phosphorylation sites (T24, S256, and S319) have been replaced by alanine residues (TSS/A-FoxO1) were crossed with female FVB/N wild type female mice (Harlan Laboratories) to generate transgenic and wild type littermate controls for studies (34). Liver-specific FoxO knockout (FoxOKO) mice in which the alleles for FoxO1, FoxO3 and FoxO4 are disrupted in the liver were generated by crossing FoxO floxed ($FoxO^{fl/fl}$) mice (from Dr. Ron DePinho) with albumin-Cre (Jackson Laboratory) mice (40). Liver-specific insulin receptor knockout (LIRKO) and IR/FoxO1 KO (LIRFKO) mice were previously generated by crossing albumin-Cre (Jackson Laboratory) with IR floxed ($IR^{fl/fl}$) (provided by CR Kahn, Harvard Medical School) and FoxO1 floxed ($FoxO1^{fl/fl}$) mice (provided by Ronald DePinho, MD Anderson Cancer Center), as previously described (25). Mice were housed on a 12-hr:12-hr 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 wild type littermates were fasted for 18 hr overnight and refed at 9 AM and sacrificed 6 hr 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 (34,40). 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 hr overnight fast, when insulin levels are low and endogenous FoxO proteins are active in the liver, as before (40).

Islet studies–For studies of β-cell proliferation, 10-week-old freely fed male LIRKO, LIRFKO, $IR^{fl/fl}$ and $IR^{fl/fl}FoxO1^{fl/fl}$ mice were sacrificed between 10 and 11 AM by decapitation following brief sedation with isoflurane. Following the collection of cervical blood, the liver was freeze-clamped with aluminum tongs pre-cooled with liquid N2 for storage at -80°C and the pancreas was fixed 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 μm thick) were probed with anti-insulin antibody (Abcam) and then scanned at 200x with an Aperio Scanscope CS for quantification of total tissue and islet cross-sectional area using ImageScope software (Aperio). For analysis of β-cell proliferation, sections were immunostained with anti-Ki67 (BD) and anti-insulin (Abcam) antibodies. Cell counting was manually performed in a blinded fashion by a single

observer. Insulin$^+$ and Ki67$^+$ beta cells were identified using fluorescence microscope (Olympus). Insulin$^+$ cells showing nuclear DAPI staining were considered as β-cells. Insulin$^+$ cells showing nuclear colocalized staining for DAPI$^+$ and Ki67$^+$ were counted as proliferating β-cells as previously described (6,17) and at least 1200 β-cells per pancreas were counted. At sacrifice, mice were briefly sedated with isofluorane prior to decapitation and liver was snap frozen in liquid nitrogen then stored at -80°C until analysis.

Hepatocytes and adenoviral vectors- As we previously described (17), hepatocytes were isolated from male mice by collagenase digestion via portal vein perfusion. Hepatocytes were washed twice in Hepatocyte Wash Medium (Invitrogen), 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 hr before further treatment. For studies in hepatocytes from LIRKO and IR$^{fl/fl}$ mice, plated cells were rinsed with phosphate buffered saline (PBS) and refed with DMEM, 25 mM glucose and 10% FBS, and harvested 24 later for ChIP analsyis. For studies of insulin effects on gene expression, plated hepatocytes from wild type C57Bl/6 mice were rinsed with PBS and then refed with DMEM plus 5 mM glucose, 0.1% FBS for 16 hours prior to treatment with DMEM containing 5 mM glucose with/without 100 nM insulin for 1-24 hr. For studies FoxO with adenoviral vectors, plated hepatocytes from wild type 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 hr when cells were refed with DMEM, 25 mM glucose, 10% FBS. Hepatocytes were harvested 24 hr later for analysis of gene expression or ChIP studies. Adenoviral vectors, which express GFP alone, or GFP plus wild type FoxO1, TSS-A FoxO1, or H215R FoxO1 were described previously (34, 41).

Chromatin Immunoprecipitation (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% of power, 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 (Lys14) (Cell Signaling #5275), anti-Acetyl-Histone H3 (Lys9) (Cell Signaling #9649) or rabbit IgG (Cell Signaling #2729), and protein/cross-linking was reversed. Normalization of relative enrichment of FoxO1 on promoter regions were 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′-ATCTGGCTAGCAGCTTGCTGA-3′ (forward) and 5′- CCGTGTGCAGTGTTCAATGCT-3′ (reverse)); G6Pase (5'-GCCTCTAGCACTCAAGCAGTG-3' (forward) and 5'- TGTGCCTTGCCCCTGTTTTATATG-3' (reverse)); PEPCK (5'-TCCACCACACACCTAGTGAGG-3' (forward) and 5'-AGGGCAGGCCTAGCCGAGACG-3' (reverse)); SerpinB1 R1P1 (5'-ACCTCCTCAAGACCTGTGGA-3' (forward) and 5'-GCTGGAGATGCCTTACCAGG-3' (reverse)); SerpinB1 R1P2 (5'-GCCAGTTACCTCCTCAAGACC-3' (forward) and 5'- CCAGGGTGGAAAGCTTAGCA-3' (reverse)); SerpinB1 R2P1 (5'-TGCTAAGCTTTCCACCCTGG-3' (forward) and 5'- CCCTCTACCCCAAGACCTCA-3' (reverse)); SerpinB1 R2P2 (5'-CCTGGTAAGGCATCTCCAGC-3' (forward) and 5'-TACCACTGAGCCCTCTACCC-3' (reverse)); SerpinB1 R3P1 (5'-CTCAGCCTCTCACCTTCTGC-3' (forward) and 5' -CTGACTTGGCCCTTGTGGAT-3' (reverse)); SerpinB1 R3P2; 5'-TCGCCATGGGACCAAAAGAA-3' (forward) and 5'-GTTCCCCTTCTGGAGCTCAC-3' (reverse)); SerpinB1 PP1 (5'-TCCTCTGAAGAAAGACAAGAAGCA-3'(forward); 5'-CAATGGCCTGGGCTAGTCAA-3'(reverse));

and SerpinB1 PP2 (5'-AGGAGAATAAAGCTGAGCCCA-3'(forward) and 5'-AATAGTGGGGAATTGTGGGTTGA-3' (reverse)).

RT-PCR– For real-time PCR experiments, total RNAs were extracted from liver or hepatocytes using Trizol method (42). One μg of total RNAs was used for a reverse transcription reaction using high-capacity cDNA Archive Kit (Applied Biosystems). cDNA was analyzed by ABI 7900HT system (Applied Biosystems). The level of TATA binding protein (TBP) mRNA transcripts was used as an internal control. Primers for SerpinB1 (5′-GCTGCTACAGGAGGCATTGC-3′ (forward) and 5′-CGGATGGTCCACTGTGAATTC-3′ (reverse)); PEPCK (5'-TGTTTACTGGGAAGG CATCG-3' (forward) and 5'-AGGTCTACGGC CACCAAAG-3' (reverse)); G6Pase (5′-ATCCGGGGCATCTACAATG-3' (forward) and 5'-TGGCAAAGGGTGTAGTGTCA-3' (reverse)); IGFBP1 (5'-GGAGATCGCCGACCTCAAG-3' (forward) and 5'-CTGCAGCTAATCTCTCTAGCACTTTAT-3' (reverse)); and TATA binding protein (5'-ACCCTTCACCAATGACTCCTATG-3' (forward) and 5'-ATGATGACTGCAGCAAATCGC-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 β-mercaptoethanol, boiled and resolved by SDS-polyacrylamide gel electrophoresis. 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 (43), GAPDH (Cell Signaling, #5174P) or mouse antiserum to actin (Santa Cruz, sc-1615) or β-tubulin (Santa Cruz, sc-5274). Secondary goat anti-rabbit (Santa Cruz, sc-2054) or goat anti-mouse (Santa Cruz, sc-2055) was used thereafter. Protein bands were quantified by ImageJ software.

Statistical Analysis–All data are presented as mean ± SEM. Data were analyzed using unpaired, two-tailed Student's t test. A p value < 0.05 was considered statistically significant.

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Author contributions: AEO, IOS, RNK and TGU conceived the idea, planned the experiments, and wrote the manuscript. AEO, IOS and GB performed the experiments and analyzed the data in LIRKO and LIRFKO mice and isolated hepatocytes. JS conducted ChIP experiments. Immunostaining studies and β-cell proliferation analysis was performed by IOS, SX, GQ and CWL. WZ and GB performed studies in FoxO1 transgenic and FoxO knockout mice. SR and TT provided technical assistance.

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Figure legends

Figure 1: β-cell proliferation and expression of serpinB1 in the liver of LIRKO and LIRFKO mice. **A**. Representative images of insulin$^+$/Ki67$^+$ cells (white arrows) in pancreases isolated from 10-week-old male IR$^{fl/fl}$ (top panel), LIRKO (middle panel) or LIRFKO (lower panel) mice. Scale bar in top panel represents 50 μm. **B**. Quantification of islet area. Data are presented as the percentage of total islet cross-sectional

area, and represent mean ± SEM; *p ≤ 0.05 *vs.* $IR^{fl/fl}$ (n = 5–8 per group). **C**. Quantification of islet mass. Data represent mean ± SEM; *p ≤ 0.05 *vs.* $IR^{fl/fl}$ (n = 5–8 per group). **D**. Quantification of $insulin^{+}/Ki67^{+}$ cells (in A). Data represent mean ± SEM; *p ≤ 0.05 (n = 5–8 per group). **E**. Insulin levels. Data represent mean ± SEM; **p ≤ 0.01 (n = 4-6 per group) **F**. Relative liver *igfbp1* mRNA levels by real-time PCR (normalized for TBP) in 10-week-old male $IR^{fl/fl}$, $IR^{fl/fl}F^{fl/fl}$, LIRKO and LIRFKO mice. Data represent mean ± SEM; **p ≤ 0.01 and ***p ≤ 0.001 (n = 5-6 per group). **G**. Relative liver *serpinb1* mRNA levels in liver of 10-week-old male $IR^{fl/fl}$, $IR^{fl/fl}F^{fl/fl}$, LIRKO and LIRFKO mice. Data represent mean ± SEM; **p ≤ 0.01 (n = 5-6 per group). **H**. Western blots of serpinB1, actin and β-tubulin in liver. **I**. Quantification of serpinB1 protein. Data represent mean ± SEM; **p ≤ 0.01 and ***p ≤ 0.001 (n = 5-6 per group).

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 hours and refed for 6 hours before livers were harvested for analysis of mRNA and protein expression. **A**. Relative liver *igfbp1* mRNA levels (normalized to TBP) in 10-week-old WT and Tgn mice. Data represent mean ± SEM; **p ≤ 0.01 (n = 3-4 per group). **B**. Relative liver *serpinb1* mRNA (normalized to TBP) in 10-week-old WT and Tgn mice. Data represent mean ± SEM; *p ≤ 0.05 (n = 3-4 per group). **C**. Western blot of serpinB1 and GAPDH in liver. **D.** Quantification of serpinB1 protein. Data represent mean ± SEM; **p ≤ 0.01 (n = 3-4 per group). **E-G.** 10-week-old male $FoxO^{fl/fl}$ and FoxOKO mice were fasted overnight for 18 hours prior to sacrifice when livers were harvested for quantification of gene and protein expression. **E**. Relative liver *igfbp1* mRNA levels by in (normalized to TBP) 10-week-old $FoxO^{fl/fl}$ and FoxOKO mice. Data represent mean ± SEM; *p ≤ 0.05 (n = 3-4 per group). **F**. Relative liver *serpinb1* mRNA levels (normalized to TBP) in 10-week-old $FoxO^{fl/fl}$ and FoxOKO mice. Data represent mean ± SEM; **p ≤ 0.01 (n = 3-4 per group). **G**. Western blot of serpinB1 and GAPDH in liver. **H**. Quantification of serpinB1 protein. Data represent mean ± SEM; *p ≤ 0.05 (n = 3-4 per group).

Figure 3: FoxO1 binds physically to sites upstream from 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(s) binding sites. **B.** Chromatin immunoprecipitation (ChIP) assay for H3K9Ac on the *gapdh* promoter in primary hepatocytes from control and LIRKO mice. **C-M.** ChIP assays for FoxO1 on *pepck*, g6pase, *igfbp1*, or *serpinb1* genomic regions in primary hepatocytes from $IR^{fl/fl}$ and LIRKO mice. Data are mean ± SEM. *P < 0.05; **p ≤ 0.01; ***p ≤ 0.001 (n = 4 per group). The ChIP qPCR was performed with the primers described in Experimental Procedures.

Figure 4: Effects of insulin and FoxO1 on serpinB1 gene expression in hepatocytes. Primary hepatocytes were isolated from 4-month-old male C57BL6/J mice were stabilized in DMEM media containing 5 mM glucose and 0.1% FBS overnight prior to treatment with (red bars)/without (blue bars) 100 nM of insulin in DMEM, 5 mM glucose for 1, 2, 4, 8, 12 or 24 hours when RNAs were extracted and *serpinb1* transcript levels were assessed by qRT-PCR. **A**. Relative quantification of hepatocyte *igfbp1* mRNA levels in presence or absence of insulin. Data represent mean ± SEM; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 (n = 6 per group). **B**. Relative quantification of liver *serpinb1* mRNA in presence (red bars) or absence (blue bars) of insulin. **C-F.** Relative quantification by qRT-PCR (normalized to Actin) of liver *igfbp1* (**C**)*, pepck* (**D**)*, g6pase* (**E**) *and serpinb1* (**F**) in mouse primary hepatocytes 40 hours after transfection at the multiplicity of infection (MOI) of 10 with adenoviruses expressing GFP (Ad-GFP), GFP plus wild type FoxO1 (Ad-FoxO1(WT)), or GFP plus constitutively active FoxO1 (Ad-FoxO1(TSS/A)). Data represent mean ± SEM; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 (n = 3 per group). G-J. Relative quantification by qRT-PCR (normalized to Actin) of liver *igfbp1* (**G**)*, Pepck* (**H**)*, g6pase* (**I**) and *serpinb1* (**J**) in hepatocytes infected for 48 hours at the MOI=100. Data represent mean ± SEM; *p ≤ 0.05; **p ≤ 0.01 (n = 3 per group).

Figure 5: FoxO1 binds to sites upstream from the *serpinB1* gene in isolated hepatocytes. Chromatin immunoprecipitation (ChIP) assay in mouse primary hepatocytes 40 hours after transfection at a multiplicity of infection of 30 with adenovirus expressing GFP (Ad-GFP), GFP plus wild type 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-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 ± SEM. *p<0.05, **p<0.01 vs GFP control; $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs WT FoxO1; $^{\$\$}$p<0.01 vs TSS-A FoxO1 (n =4 per group). The ChIP qPCR was performed with the primers described in Experimental Procedures.

Figure 1: Regulation of serpinB1 in the liver of LIRKO and LIRFKO mice

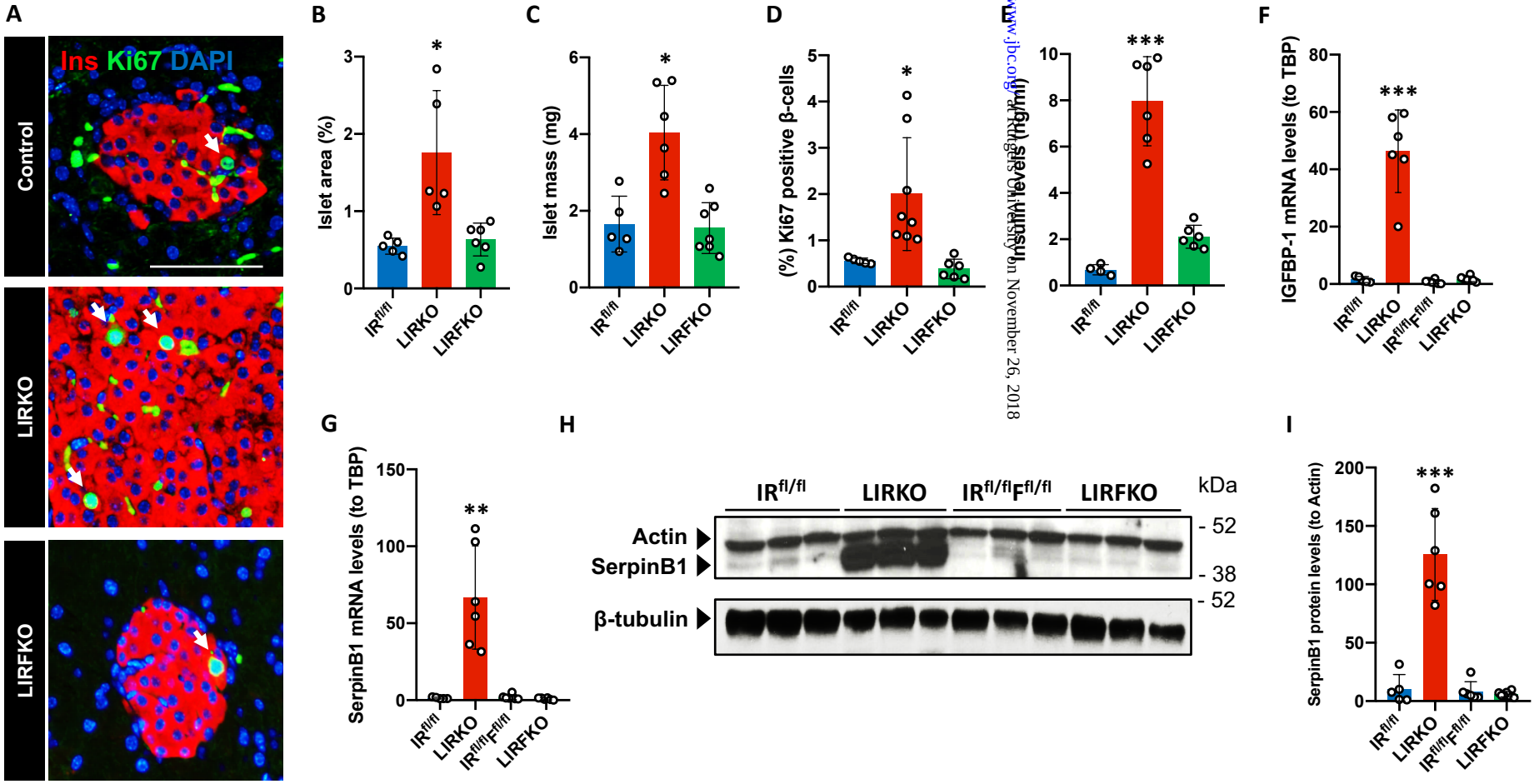


Figure 2: Regulation of hepatic serpinB1 by FoxO proteins *in vivo*

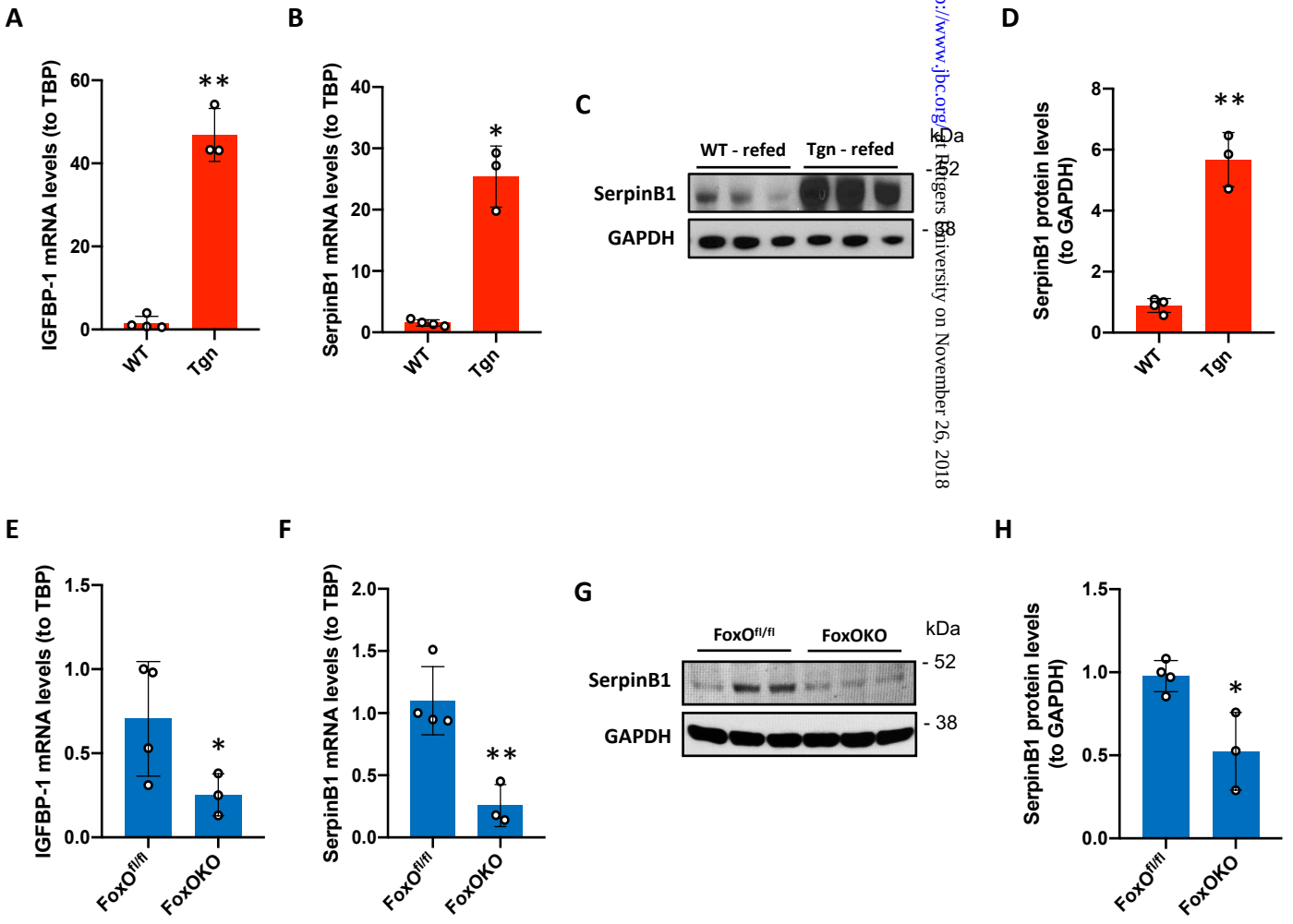


Figure 3: FoxO1 binds physically to *serpinb1* promoter regions

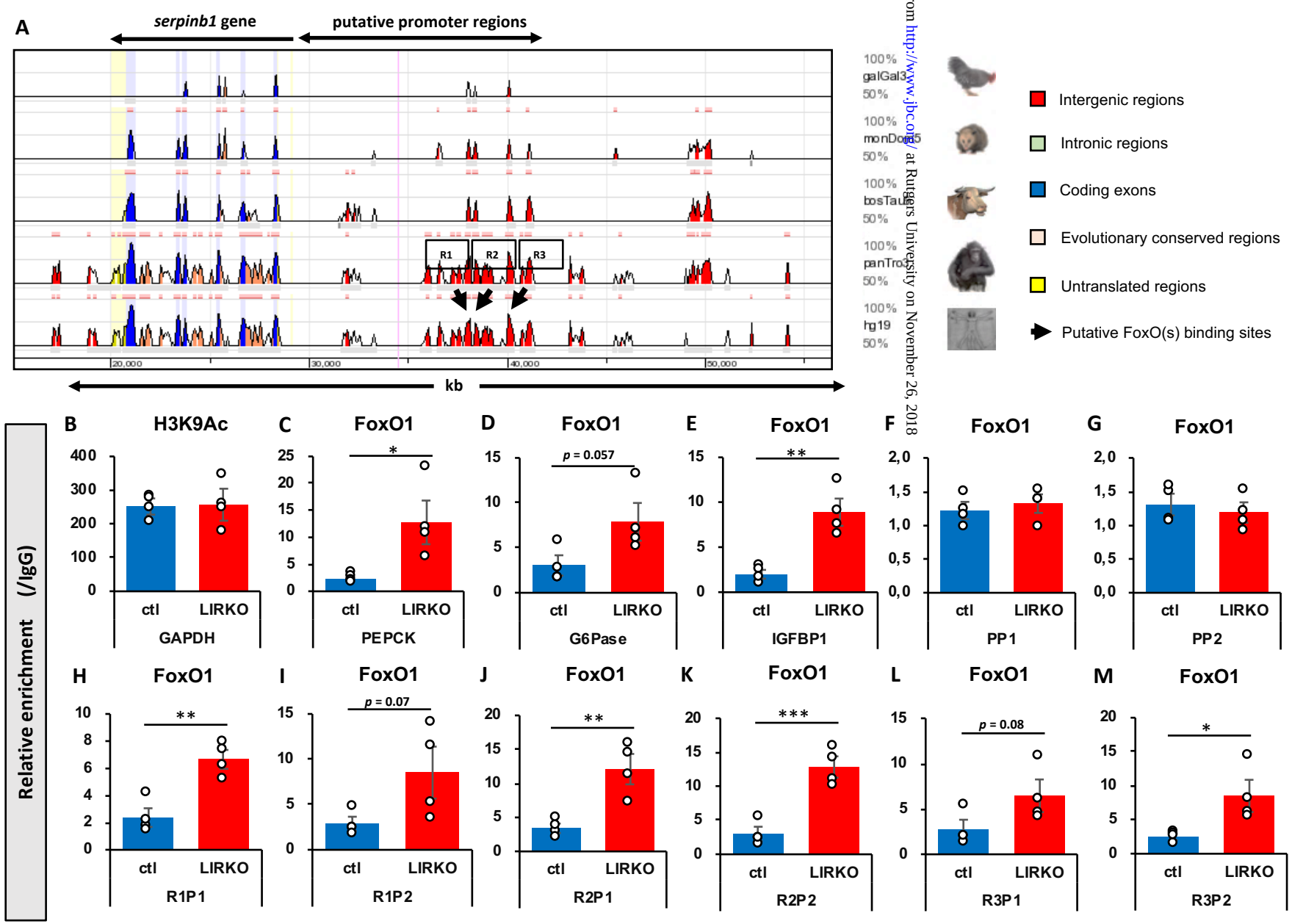


Figure 4: Effects of insulin and FoxO1 on serpinB1 gene expression in hepatocytes

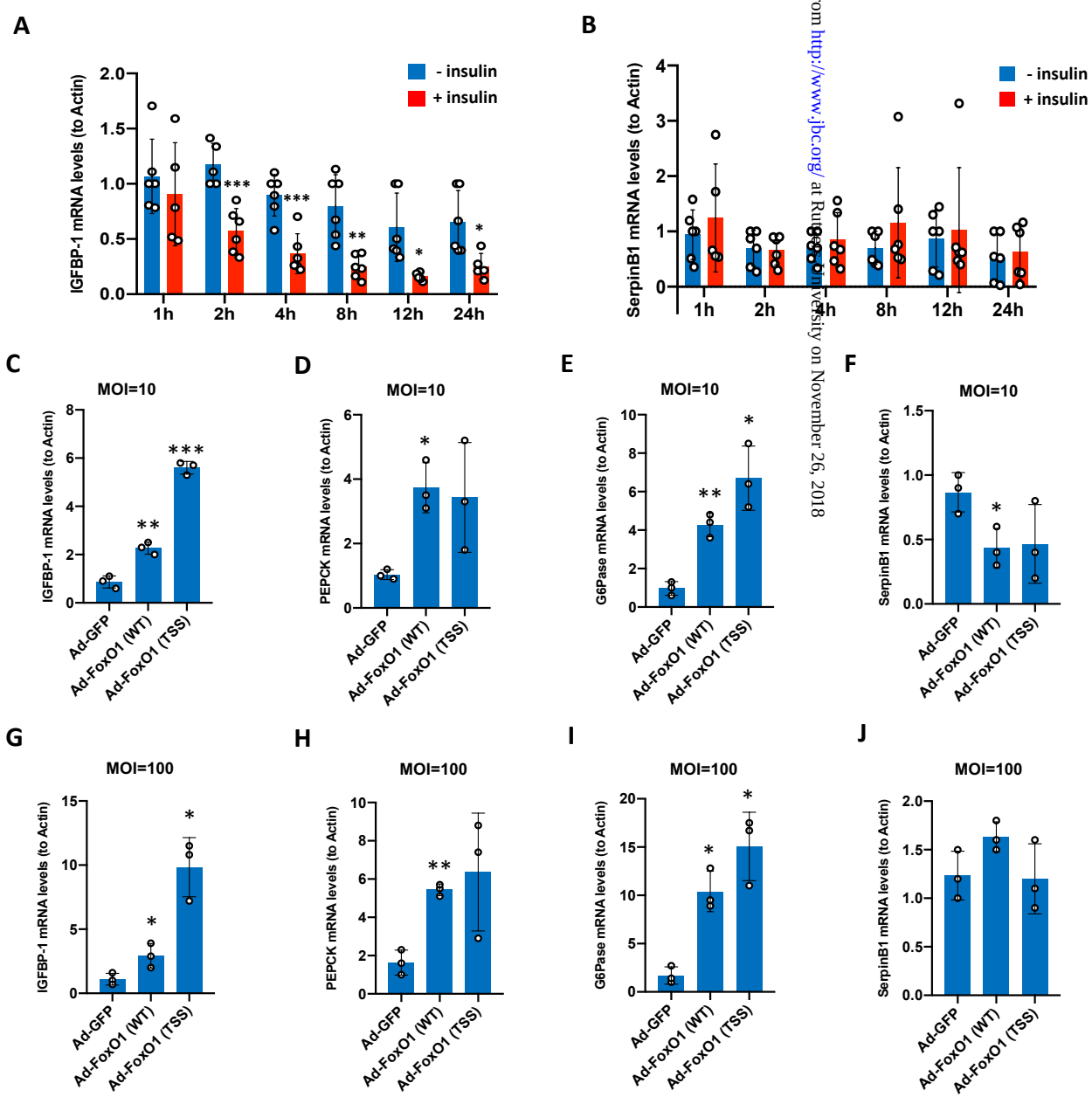
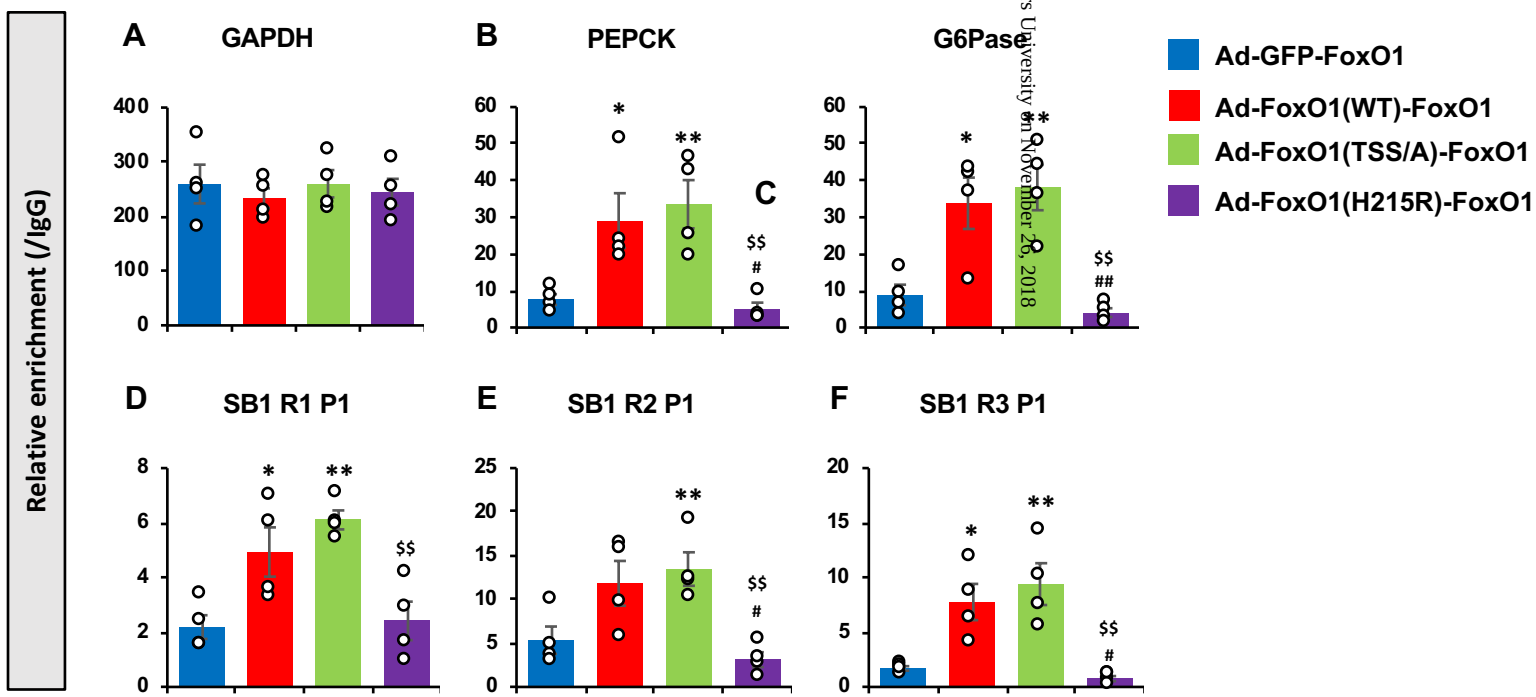


Figure 5: The overexpression of FoxO1 does not affect the binding to SerpinB1 upstream sites in hepatocytes



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

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