Hepatitis B X-interacting protein promotes the formation of the insulin gene–transcribing protein complex Pdx-1/Neurod1 in animal pancreatic β-cells

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The activation of insulin gene transcription depends on multiple nuclear proteins, including the transcription factors PDX-1 and NEUROD1, which form a transcriptional complex. We recently reported that hepatitis B X-interacting protein (HBXIP, also termed LAMTOR5) can modulate glucose metabolism reprogramming in cancer cells. However, the physiological role of HBXIP in the modulation of glucose metabolism in normal tissues is poorly understood. Here, we report that Hbxip is an essential regulator of the effect of the Pdx-1/Neurod1 complex on insulin gene transcription in murine pancreatic β-cells *in vitro* and *in vivo*. We found that pancreatic β-cell–specific Hbxip-knockout mice displayed higher fasting blood glucose levels and impaired glucose tolerance. Furthermore, Hbxip was involved in the regulation of insulin in the pancreas islets and increased insulin gene expression in rat pancreatic β-cells. Mechanistically, Hbxip stimulated insulin enhancer activity by interacting with Pdx-1 and recruiting Neurod1 to Pdx-1. Functionally, we provide evidence that Hbxip is required for Pdx-1/Neurod1-mediated insulin expression in rat pancreatic β-cells. Collectively, these results indicate that Hbxip is involved in the transcription of insulin by increasing the levels of the Pdx-1/Neurod1 complex in animal pancreatic β-cells. Our finding provides the insight into the mechanism by which Hbxip stimulates the transcription of the insulin gene.

Production and secretion of insulin from β-cells in the pancreas are crucial for maintaining normoglycemia (1, 2). Insulin is secreted immediately in response to increased glucose levels, and the same dynamic response has been shown for the biosynthesis at the levels of transcription and translation (3–5). The activation of insulin gene transcription depends on multiple nuclear proteins that build a transcription activation complex (6, 7). Two β-cell–specific transcription regulators, PDX-1 (pancreatic and duodenal homeobox-1) and NEUROD1 (neurogenic differentiation 1), have been demonstrated to play crucial roles in glucose-induced insulin gene transcription and pancreatic β-cell function (8, 9). The previous research provides evidence that the homeodomain of Pdx-1 acts as a protein–protein interaction domain to recruit multiple proteins, including Neurod1, basic helix-loop-helix protein E47/Pan1, and high-mobility– group protein I (Y), to an activated complex on the insulin E2A3/4 mini-enhancer during the transcription of insulin (10). However, the underlying mechanism by which the transcription factor complex Pdx-1/Neurod1 modulates insulin expression is not well documented.

Hepatitis B X-interacting protein (HBXIP,[3] also termed LAMTOR5) is a cellular 18-kDa protein among mammalian species, which was originally named by identifying binding partners of hepatitis B virus X protein in hepatoma HepG2 cells (11). A previous study indicates that HBXIP is required for amino acid sensing via the mTORC1 pathway in cell growth (12). For many years our group has been focusing on the carcinogenicity of HBXIP as a transcription co-activator to activate multiple target genes during the progression of cancers. We have reported that HBXIP plays a crucial role in the development of cancer by acting as a co-activator of transcription factors, such as c-Myc, liver X receptors, TFIID, and MDM2 (13–17). Recently, our study has indicated that HBXIP promotes glucose metabolism reprogramming for breast cancer growth through modulating SCO2, PDHA1, or PCK1 (18, 19). However, the physiological role of HBXIP in modulation of glucose metabolism in normal tissues is poorly understood.

In this study, we first found that HBXIP was highly expressed in pancreas islets compared with other cells in the pancreas. Because the insulin produced from pancreatic β-cells was thought to play an important role in regulation of the blood glucose level, we are interested in the physiological significance of HBXIP as a co-activator in the transcriptional regulation of insulin, which is the key gene for maintaining normoglycemia

[3] The abbreviations used are: HBXIP, hepatitis B X-interacting protein; IHC, immunohistochemistry; HE, hematoxylin-eosin; NCD, normal chow diet; HFD, high-fat diet; IGPPT, intraperitoneal glucose tolerance test; IVGTT, intravenous glucose tolerance test; GSIS, glucose-stimulated insulin secretion; co-IP, co-immunoprecipitation.
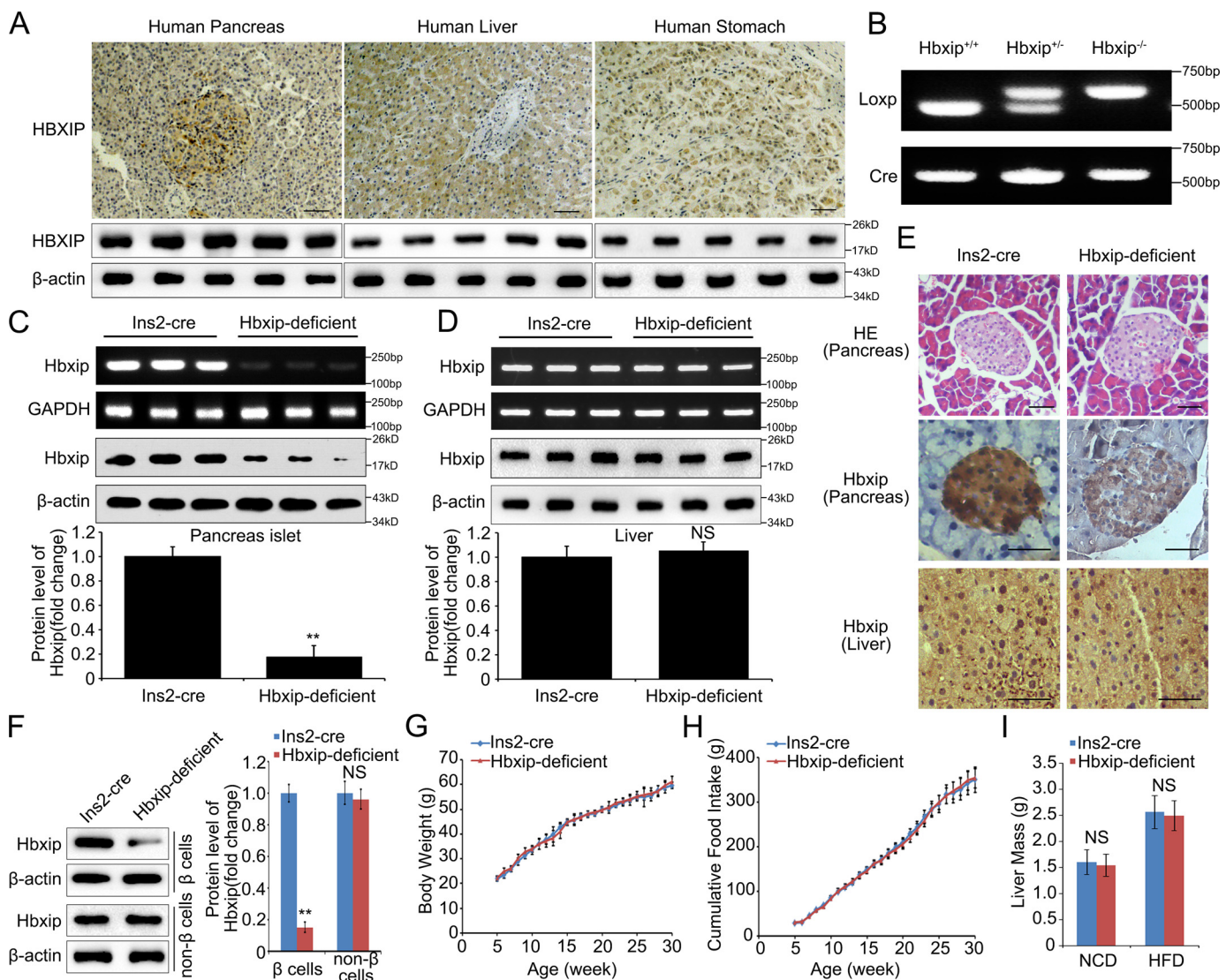
Figure 1. Pancreatic β-cell–specific Hbxip-knockout mice are generated. *A,* expression level of HBXIP was tested in pancreas, liver, and stomach tissues of human by IHC staining. *Scale bars,* 80 μm. Representative figure was presented from the analyses of five different persons per group. The protein level of HBXIP between human pancreas, liver, and stomach of five different persons was examined by Western blot analysis. *B,* identification of Ins2-cre (wildtype), Hbxip$^{+/-}$, and Hbxip-deficient mice. *C* and *D,* relative mRNA and protein levels of Hbxip were examined in the pancreas islet and liver tissues of Ins2-cre and Hbxip-deficient mice. The protein levels of Hbxip from different groups of mice were quantified using ImageJ software as shown. Mean ± S.D., $n = 3$/group. Student's *t* test; **, $p < 0.01$. *E,* pancreatic morphology was examined by HE staining in the pancreas tissues of Ins2-cre and Hbxip-deficient mice. The expression levels of Hbxip were examined by IHC staining in the pancreas and liver tissues of Ins2-cre and Hbxip-deficient mice. *Scale bars,* 80 μm. Representative figures were presented from the analyses of five different mice per group. *F,* expression of Hbxip was measured by Western blot analysis in pure β- and non-β-cells obtained from flow cytometry using islets isolated from Ins2-cre and Hbxip-deficient mice. The protein levels of Hbxip from different groups of mice were quantified using ImageJ software as shown. Mean ± S.D., $n = 3$/group. Student's *t* test; **, $p < 0.01$. *NS,* not significant. *G,* body weight change on HFD. Mean ± S.D., $n = 6$/group. *H,* cumulative food intake on HFD. Food intake was measured from three different cages, and two mice were housed in each cage. Mean ± S.D. *I,* liver mass on NCD or HFD. Mean ± S.D., $n = 6$/group. *NS,* not significant. Each experiment was repeated at least three times.

in this study. Interestingly, we showed that Hbxip was involved in the glucose-induced insulin gene transcription through a model of pancreatic β-cell–specific Hbxip-knockout mice. Our finding provides the insight into the mechanism by which Hbxip physiologically modulates the transcription of the insulin gene.

Results

Pancreatic β-cell–specific Hbxip-knockout mice are generated

To better understand the physiological significance of HBXIP, we first detected the expression level of HBXIP in human normal tissues. Our group has previously revealed the

HBXIP levels in a variety of normal tissues (17). Here, we found that HBXIP was ubiquitously expressed in most normal tissues (Fig. 1*A* and Fig. S1*A*). Interestingly, Western blot analysis showed that among the tissues linked with metabolism, the expression level of HBXIP was higher in the pancreas than in the liver and stomach (Fig. 1*A*). Meanwhile, HBXIP was highly expressed in pancreas islets compared with other parts of pancreas as shown in the staining results (Fig. 1*A*). The above data implied that HBXIP might play a crucial role in pancreas islets. Then, we constructed an Hbxip-knockout allele using the Cre-loxP approach (20, 21) and generated pancreatic β-cell–specific Hbxip-knockout mice (Hbxip-deficient mice) accord-

ing to previous research (22). Because the Ins2-Cre mice alone displayed glucose intolerance (23), the Ins2-cre littermates were used as wildtype controls. The mice were verified via PCR analysis, and our result showed that the floxed allele gave rise to a PCR product ~40 bp larger than the Ins2-cre (wildtype) allele PCR product (Fig. 1*B*). Our data showed that the mRNA and protein levels of Hbxip in pancreas islets were decreased in Hbxip-deficient mice relative to those of Ins2-cre mice (Fig. 1*C*). Meanwhile, the expression level of Hbxip had no distinction in the liver from Ins2-cre and Hbxip-deficient mice (Fig. 1*D*). Additionally, we observed the morphology of islets through hematoxylin and eosin (HE) staining, and the lower levels of Hbxip were validated by immunohistochemistry (IHC) staining in the pancreas of Hbxip-deficient mice (Fig. 1*E*). Moreover, the Hbxip levels showed no differences in the liver and in some other tissues of Ins2-cre and Hbxip-deficient mice (Fig. 1*E* and Fig. S1*B*). Therefore, the knockout of Hbxip was completed specifically in the pancreas of Hbxip-deficient mice. To quantitatively show how effectively Hbxip was removed from pancreatic β-cells, we examined the protein level of Hbxip in pure β-cells, as well as non-β-cells, obtained from the islets of Ins2-cre and Hbxip-deficient mice using flow cytometry (24). The result indicated that the expression level of Hbxip was decreased noticeably in β-cells from Hbxip-deficient mice, whereas no significant difference was observed between the non-β-cells of two mice models (Fig. 1*F*), further suggesting that Hbxip was markedly deleted from islet β-cell population by Ins2-Cre mice. In addition, the Hbxip-deficient mice exhibited normal body weight gain, food intake on high-fat diet (HFD), and normal liver mass either on normal chow diet (NCD) or HFD (Fig. 1, *G–I*), supporting that the pancreas-specific knockout of Hbxip had no side effect on the Hbxip-deficient mice compared with the Ins2-cre mice. Additionally, we found that Hbxip was expressed little in the brain of model mice, and the difference of Hbxip expression between Ins2-cre and Hbxip-deficient mice brain was not significant (Fig. S1*C*). Therefore, the effect of the leaky expression of Cre in the model mouse brain (25) on the phenotype of the Hbxip-deficient mice was thought to be ignorable. Thus, the model of pancreatic β-cell–specific Hbxip-knockout mice was successfully established.

Hbxip is involved in the regulation of insulin in pancreas islets of mice

Next, we observed the phenotypes of Hbxip-deficient mice in glucose tolerance and insulin production. Interestingly, both NCD and HFD Hbxip-deficient mice, as opposed to Ins2-cre mice, exhibited higher blood glucose levels in fasting conditions (Fig. 2*A*). Furthermore, both NCD and HFD Hbxip-deficient mice developed glucose intolerance compared with Ins2-cre mice upon intraperitoneal glucose administration, and this effect was exacerbated in the obese state (Fig. 2*B*). Despite glucose intolerance, these mice exhibited normal insulin sensitivity, as shown by insulin tolerance testing (Fig. 2*C*), suggesting that a defect in insulin production caused hyperglycemia. Moreover, we examined circulating insulin levels during the intraperitoneal glucose tolerance tests (IPGTTs). The NCD and HFD Hbxip-deficient mice displayed decreased insulin levels compared with their Ins2-cre counterparts (Fig. 2*D*), indicating

that Hbxip deficiency in the pancreas resulted in a β-cell insulin production defect. Meanwhile, we validated the glucose intolerance and the defect in insulin secretion in the Hbxip-deficient mice by intravenous glucose tolerance tests (IVGTTs) (Fig. 2, *E* and *F*). Given that the β-cell dysfunction is largely manifested as impaired glucose-stimulated insulin secretion (GSIS) (26), we measured insulin secretion under static incubation conditions by isolated islets obtained from NCD and HFD Ins2-cre and Hbxip-deficient mice. The result showed that the islets of Hbxip-deficient mice exhibited a marked decrease in GSIS that was more profound in islets obtained from HFD Hbxip-deficient mice (Fig. 2, *G* and *H*). Moreover, we performed the GSIS test using the isolated Hbxip floxed islets treated with adeno-LacZ control vector or adeno-Cre vector according to a previous report (27). The result also revealed that the islets treated with adeno-Cre showed a lower level of GSIS in NCD and HFD mice (Fig. 2, *I* and *J*), which further supported that the leaky effect of Cre in the brain on the model mice could be ignored. The reduced protein level of insulin in the Ad-Cre-treated Hbxip floxed islets was also validated by Western blot analysis (Fig. 2, *I* and *J*). In addition, IHC staining showed that the levels of insulin expression were lower in the pancreas of Hbxip-deficient mice relative to Ins2-cre mice (Fig. 2*K*). Furthermore, the insulin content in the isolated islets from Hbxip-deficient mice was decreased compared with the islets of Ins2-cre mice (Fig. 2*L*). Thus, our results reveal that Hbxip-deficient mice display the phenotypes of impaired glucose tolerance and reduced insulin production, suggesting that Hbxip is involved in the regulation of insulin in the pancreas islets of mice.

Hbxip is able to promote insulin transcription in rat pancreatic β-cells

Because our previous reports have revealed that HBXIP can function as a co-activator to enhance the activities of transcription factors for the transcription activation of many genes, we further investigated the effect of Hbxip on the insulin expression in β-cells. Given that rat pancreatic β-cell INS-1 is commonly used as a model of insulin secretion (28, 29), the insulin level of cell culture supernatant is able to indicate the expression level of insulin in the cells. We found that overexpression of Hbxip resulted in the increase of insulin in the culture supernatant of INS-1 cells in a dose-dependent manner (Fig. 3*A*), and knockdown of Hbxip by siRNA led to the opposite results in the system (Fig. 3*B*), suggesting that Hbxip could up-regulate insulin expression in rat pancreatic β-cells. Moreover, overexpression of Hbxip up-regulated two transcripts of insulin (insulin1 and insulin2) in INS-1 cells in a dose-dependent manner (Fig. 3*C*). In contrast, deletion of Hbxip could down-regulate insulin at mRNA levels in the cells (Fig. 3*D*). The result was confirmed at the protein levels (Fig. 3, *E* and *F*). It has been reported that a short portion of the rat insulin promoter between bp −247 and −197 upstream from the transcription initiation site has been used as a model of the types of synergistic interactions that combine to give the characteristic activity of the full promoter (10). Accordingly, we cloned the short portion of the insulin promoter, which was also called the E2A3/4 mini-enhancer, into the pGL3-Basic plasmid and named it *Ins* mini-enhancer (Fig. 3*G*). Then, we showed that Hbxip could increase the activ-
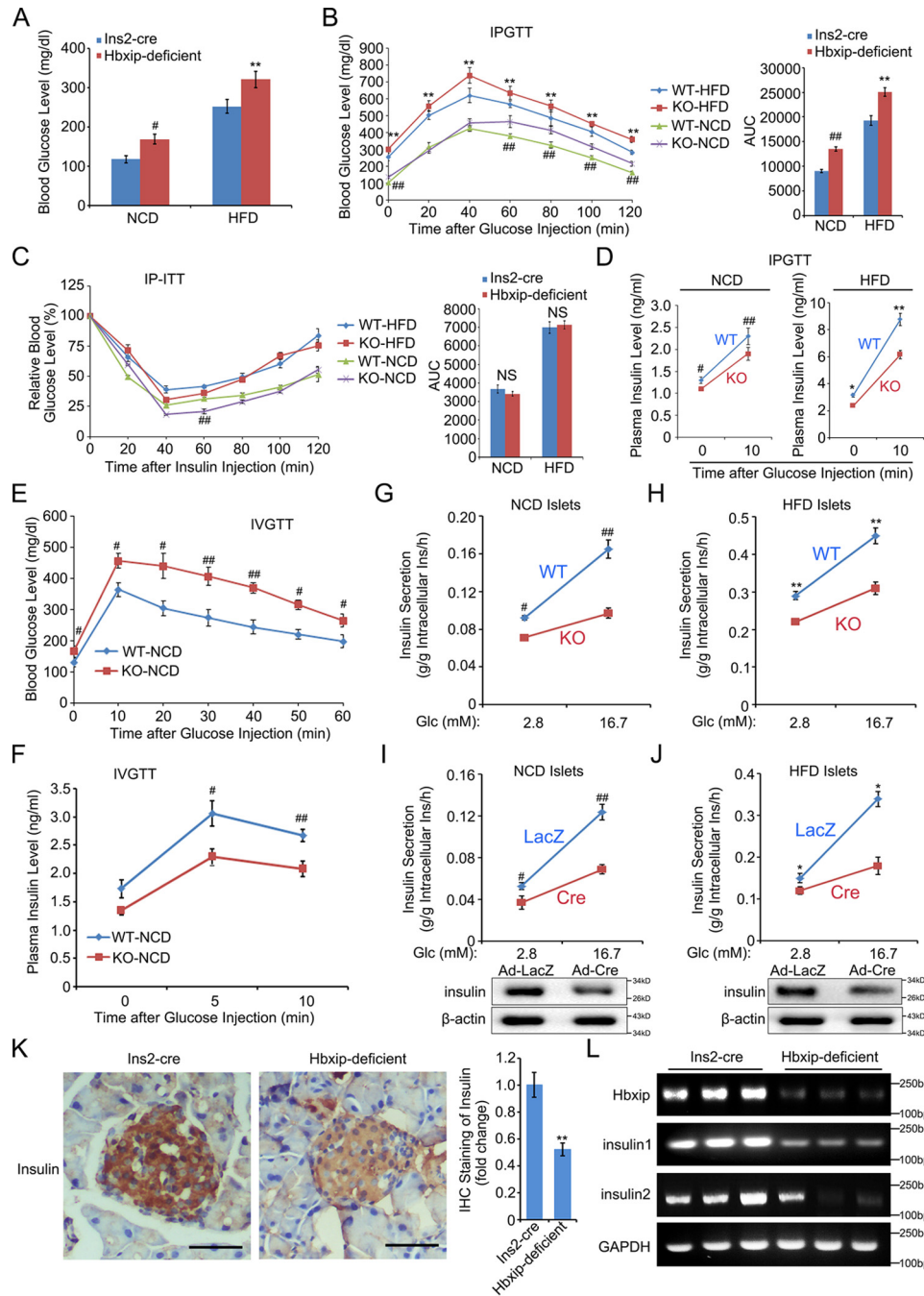
Figure 2. Hbxip is involved in the regulation of insulin in pancreas islets of mice. *A–H,* WT-NCD, Ins2-cre-NCD; KO-NCD, Hbxip-deficient-NCD; WT-HFD, Ins2-cre-HFD; KO-HFD, Hbxip-deficient-HFD. *A,* blood glucose level was examined by using Bayer Brand glucometer in Ins2-cre and Hbxip-deficient mice under fasting conditions (*n* = 6/group, 16-h fasting). Mean ± S.D. #, *p* < 0.05 WT-NCD *versus* KO-NCD; **, *p* < 0.01 WT-HFD *versus* KO-HFD. *B,* intraperitoneal glucose tolerance test. Mean ± S.D., *n* = 6/group. ##, *p* < 0.01 WT-NCD *versus* KO-NCD; **, *p* < 0.01 WT-HFD *versus* KO-HFD. Area under the curve (*AUC*) is shown in the *right panel*. *C,* insulin tolerance test. Mean ± S.D., *n* = 6/group. ##, *p* < 0.01 WT-NCD *versus* KO-NCD; *NS,* not significant. Area under the curve is shown in the *right panel*. *D,* plasma insulin levels of NCD and HFD mice during IPGTT in *A*. Mean ± S.D. #, *p* < 0.05 WT-NCD *versus* KO-NCD; ##, *p* < 0.01 WT-NCD *versus* KO-NCD; *, *p* < 0.05 WT-HFD *versus* KO-HFD; **, *p* < 0.01 WT-HFD *versus* KO-HFD. *E* and *F,* intravenous glucose tolerance test (*E*) and plasma insulin level during IVGTT (*F*). Mean ± S.D., *n* = 6/group. #, *p* < 0.05; ##, *p* < 0.01 WT-NCD *versus* KO-NCD. *G* and *H,* static GSIS test using primary mouse islets from Ins2-cre and Hbxip-deficient mice fed NCD or HFD for 10 weeks. Mean ± S.D., *n* = 6/group. #, *p* < 0.05 WT-NCD *versus* KO-NCD; ##, *p* < 0.01 WT-NCD *versus* KO-NCD; **, *p* < 0.01 WT-HFD *versus* KO-HFD. *I* and *J,* islets from floxed Hbxip mice fed NCD or HFD for 10 weeks were transduced with adeno-LacZ or adeno-Cre. Twenty four hours later, static GSIS test was performed using the islets. Mean ± S.D., *n* = 6/group. #, *p* < 0.05 LacZ-NCD *versus* Cre-NCD; ##, *p* < 0.01 LacZ-NCD *versus* Cre-NCD; *, *p* < 0.05 LacZ-HFD *versus* Cre-HFD. The expression level of insulin was tested by Western blot analysis in islets. *K,* the expression level of insulin was tested in the pancreas tissues of Ins2-cre and Hbxip-deficient mice by IHC staining. *Scale bars,* 80 μm. The results were quantified using ImageJ software as shown. Mean ± S.D. Student's *t* test; **, *p* < 0.01. *L,* insulin content of isolated islets from Hbxip-deficient mice and Ins2-cre mice. The expressions of Hbxip, insulin1, and insulin2 at the level of mRNA were examined by RT-PCR in pancreas islet tissues from Ins2-cre and Hbxip-deficient mice. Each experiment was repeated at least three times.
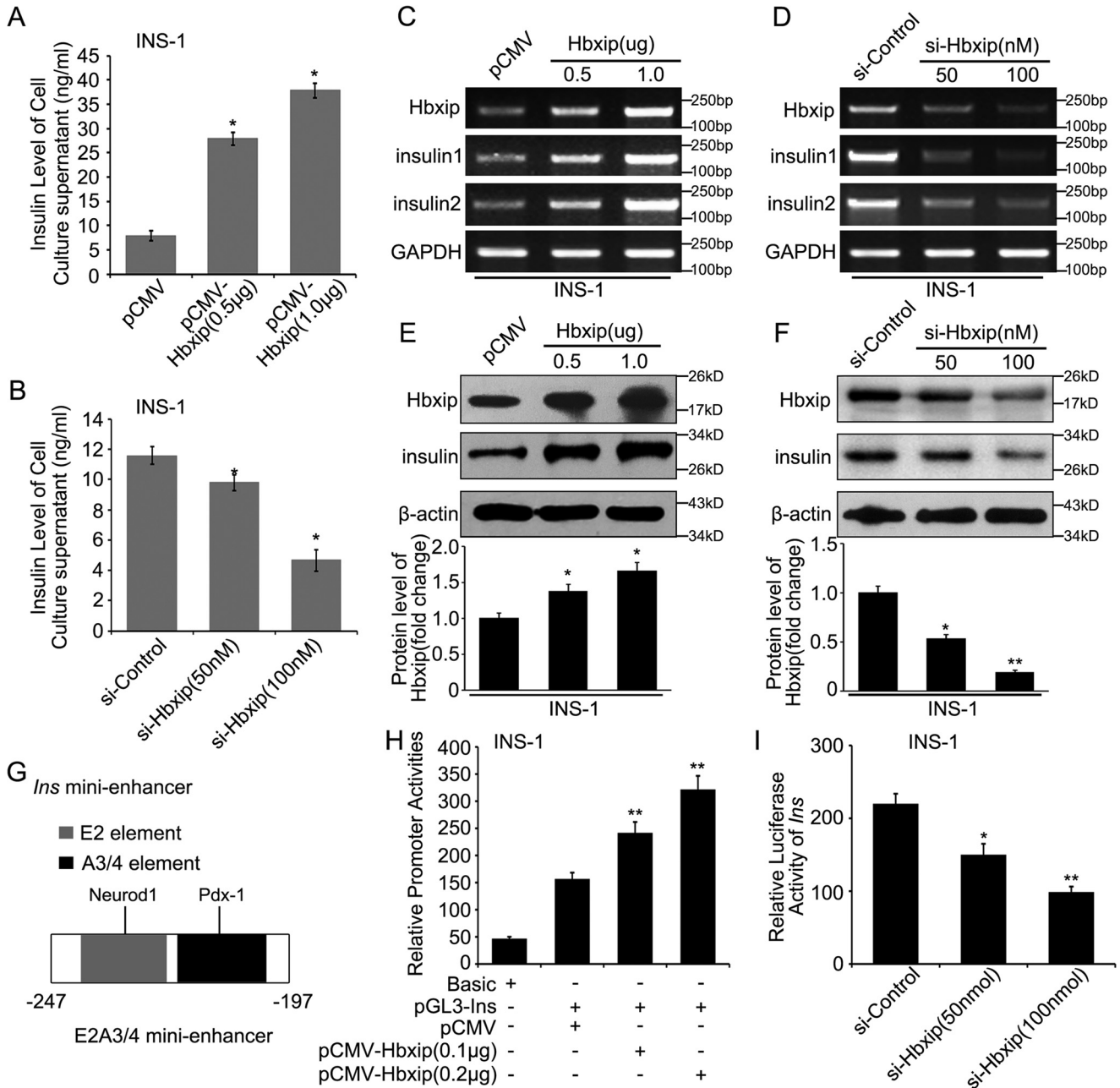
Figure 3. Hbxip is able to promote insulin transcription in rat pancreatic β-cells. *A* and *B,* insulin levels of the culture supernatant of INS-1 cells were examined by insulin assay kit. *C* and *D,* expressions of Hbxip, insulin1, and insulin2 were examined by RT-PCR in INS-1 cells at the level of mRNA, respectively. *E* and *F,* expressions of Hbxip and insulin were measured by Western blot analysis in INS-1 cells at the level of protein, respectively. The protein levels of Hbxip from three independent experiments were quantified using ImageJ software as shown. *G,* E2A3/4 mini-enhancer of insulin promoter is shown. *H,* relative mini-enhancer activities were detected by luciferase reporter gene assays in INS-1 cells transfected with pCMV-Hbxip plasmids. *I,* effect of Hbxip siRNA on the activities of *Ins* mini-enhancer was determined by luciferase reporter gene assays in INS-1 cells. Mean ± S.D. Each experiment was repeated at least three times. Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

ities of the *Ins* mini-enhancer in INS-1 cells (Fig. 3*H*). Conversely, the silencing of Hbxip attenuated the activities of *Ins* mini-enhancer in the cells (Fig. 3*I*). Thus, we conclude that Hbxip is able to promote insulin transcription in rat pancreatic β-cells.

Hbxip co-activates the transcription factor Pdx-1 to stimulate the mini-enhancer of insulin

According to previous research, the transcription level of insulin is regulated by a variety of regulators, especially the transcription factor Pdx-1, which also interacts with the A3/4 element of the mini-enhancer (10, 30, 31). Thus, we cloned the construct of *Ins* mini-enhancer with a mutant Pdx-1 element (termed pGL3-Ins-mut) (Fig. 4*A*) and found that Hbxip failed to activate pGL3-Ins-mut in the cells (Fig. 4*B*), suggesting that Hbxip might activate *Ins* mini-enhancer through transcription factor Pdx-1. Moreover, overexpression of Hbxip failed to work when Pdx-1 was silenced (Fig. 4*C*). The interference efficiency of Pdx-1 siRNA was validated by Western blot analysis in INS-1 cells (Fig. 4*D*). In addition, the knockdown of Hbxip or HBXIP
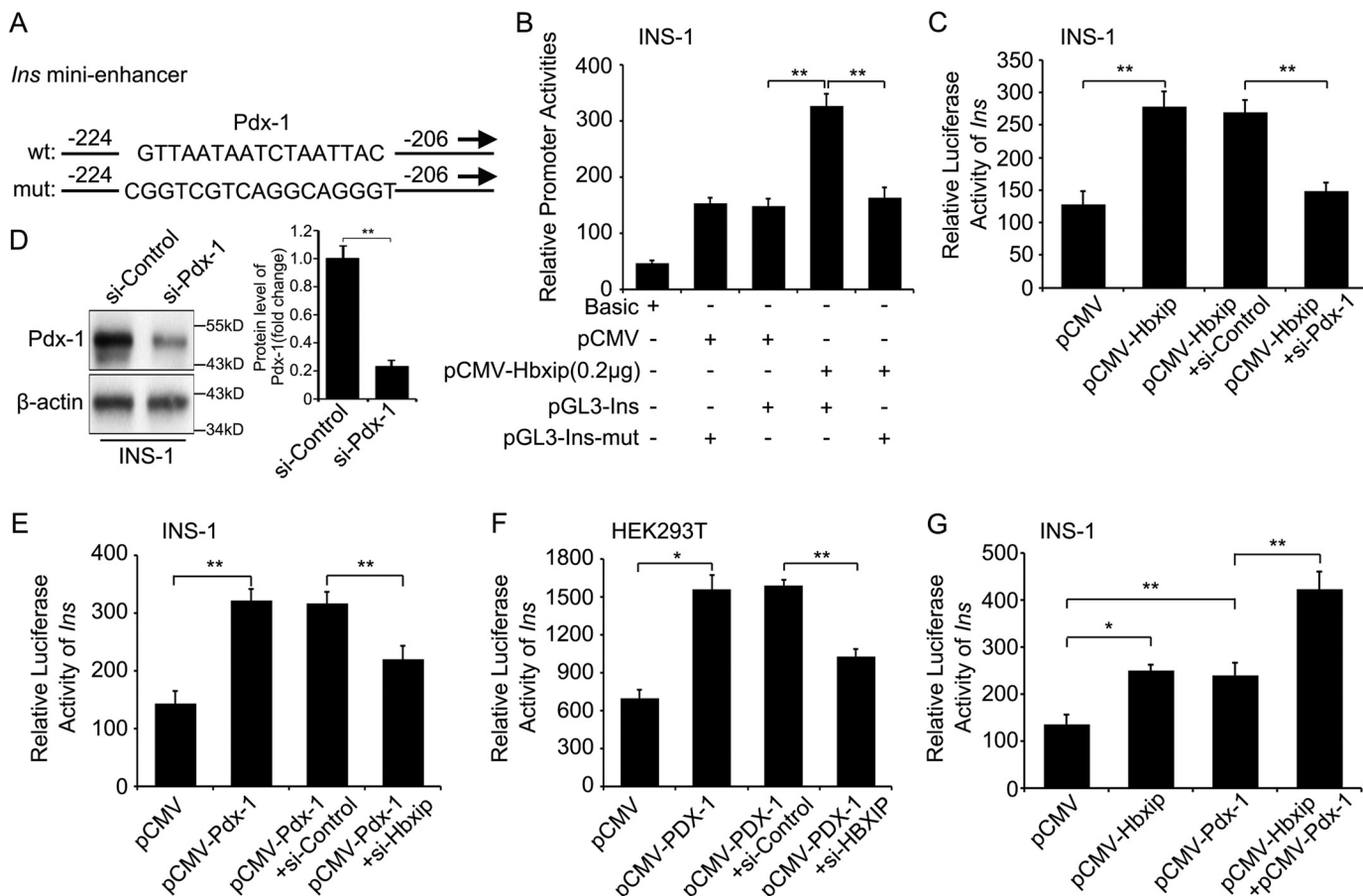
Figure 4. Hbxip co-activates the transcription factor Pdx-1 to stimulate the mini-enhancer of insulin. *A,* mutation of the Pdx-1 element is shown in the *Ins* mini-enhancer. *B,* luciferase activities were examined by luciferase reporter gene assays in the wildtype *Ins* mini-enhancer or mutant *Ins* mini-enhancer with mutant sites of Pdx-1 in INS-1 cells. *C,* luciferase activities of the *Ins* mini-enhancer were detected by luciferase reporter gene assays in INS-1 cells transfected with pCMV-Hbxip and Pdx-1 siRNA. *D,* interference efficiency of Pdx-1 siRNA was texted by Western blot analysis in INS-1 cells. The protein levels of Pdx-1 from three independent experiments were quantified using ImageJ software as shown. *E* and *F,* luciferase activities of the *Ins* mini-enhancer were measured by luciferase reporter gene assays in INS-1 and HEK293T cells transfected with relative plasmids and siRNAs. *G, Ins* mini-enhancer activities were measured by luciferase reporter gene assays in INS-1 cells transfected with pCMV-Hbxip and/or pCMV-Pdx-1 plasmids. Mean ± S.D. Each experiment was repeated at least three times. Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

markedly attenuated the increased activities of *Ins* mini-enhancer mediated by Pdx-1 or PDX-1 in INS-1 or HEK293T cells (Fig. 4, *E* and *F*), supporting that Hbxip stimulated *Ins* mini-enhancer through activating the transcription factor Pdx-1. Moreover, both overexpression of Hbxip and Pdx-1 increased the *Ins* mini-enhancer activities relative to single use in INS-1 cells (Fig. *4G*). Thus, Hbxip stimulates the enhancer of insulin through co-activating the transcription factor Pdx-1.

Hbxip forms a complex with Pdx-1 and Neurod1 in the insulin mini-enhancer in pancreatic β-cells

Next, we tried to validate the interaction of Hbxip with Pdx-1 during insulin transcription. As expected, confocal images showed that most of Hbxip and Pdx-1 co-localized in the nuclei of INS-1 cells (Fig. 5*A*), implying that Hbxip could interact with Pdx-1. Moreover, co-IP assays confirmed that Hbxip could bind to Pdx-1 and vice versa (Fig. 5*B*). GST pulldown assays indicated that Hbxip directly interacted with Pdx-1 *in vitro* (Fig. 5*C*). To identify the Pdx-1-binding domain for Hbxip, we proceeded to divide the full-length Pdx-1 into three fragments according to previous a report (Fig. 5*D*) (10). Interestingly, the

fragment Pdx-1–2 (amino acids 138–213), including the homeodomain, could directly bind to Hbxip, rather than others (Fig. 5*E*), suggesting that Hbxip directly interacted with the Pdx-1–2. Furthermore, ChIP assays revealed that Hbxip could occupy the *Ins* mini-enhancer, but Pdx-1 siRNA could block the occupation (Fig. 5*F*). Meanwhile, we chose a short portion between bp −500 and −350 upstream from the transcription initiation site of the insulin gene, which was without Pdx-1-binding sites, as negative control regions according to Ref. 31. The result showed that Hbxip could not bind to the negative control regions (Fig. 5*F*). These results suggest that Hbxip occupies the *Ins* mini-enhancer via Pdx-1 as indicated in Fig. 5*G*.

Additionally, the Neurod1 functioning in the E2 element of the *Ins* mini-enhancer is crucial for the activity of Pdx-1 by forming a dimer with Pdx-1 during the transcription of insulin (8, 32). Therefore, we were concerned whether Neurod1 was also involved in the insulin transcription induced by Hbxip. Co-IP assays revealed that Neurod1 was involved in the complex of Hbxip and Pdx-1 (Fig. 5*H*), suggesting that Hbxip, Neurod1, and Pdx-1 formed a complex in pancreatic β-cells. Then, silencing of Hbxip could attenuate the interaction of Pdx-1 with
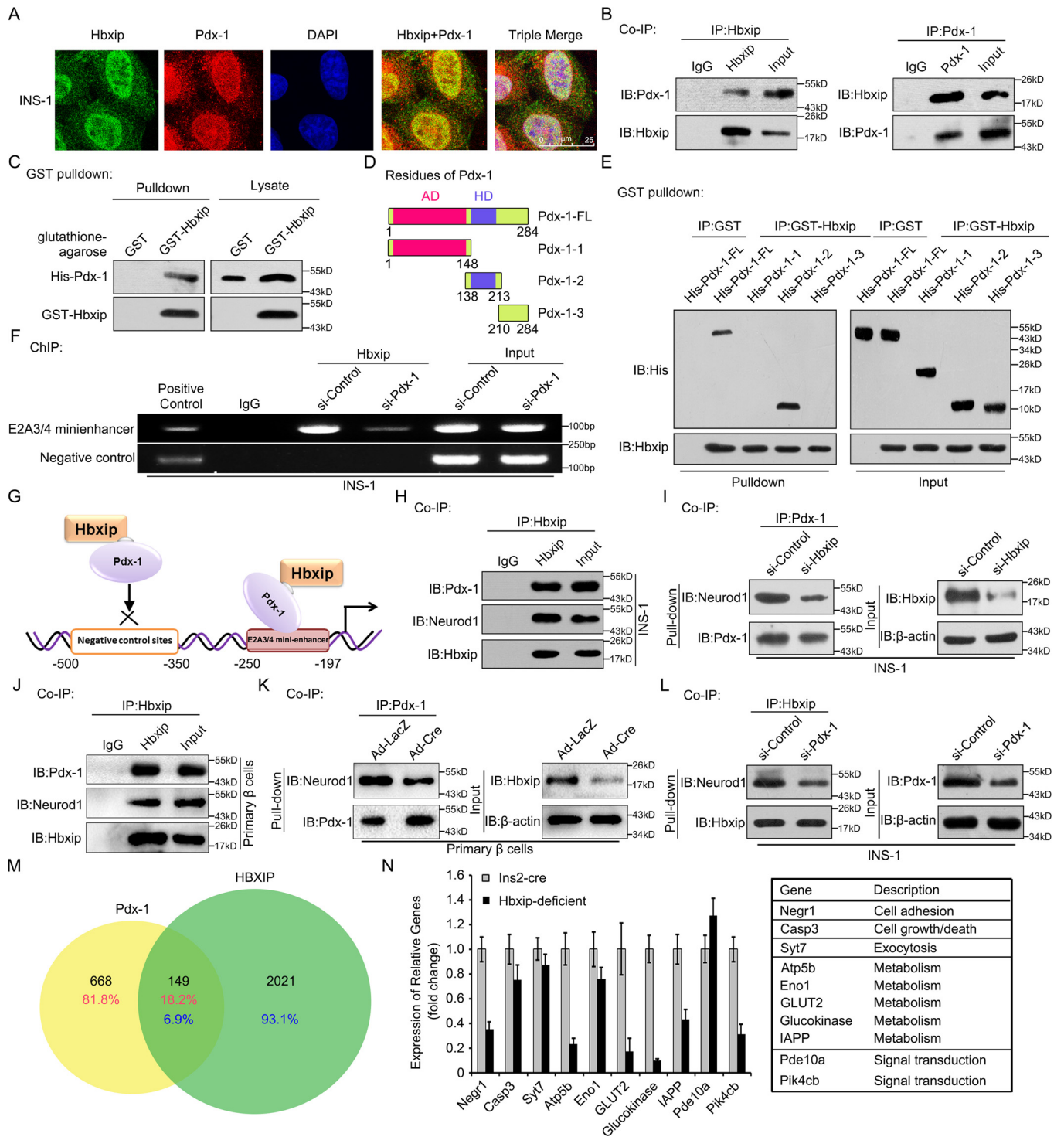
Figure 5. Hbxip forms a complex with Pdx-1 and Neurod1 in the insulin mini-enhancer in pancreatic β-cells. *A,* localization of both GFP-Hbxip and Pdx-1 was observed by confocal microscopy in the INS-1 cells. *Scale bar,* 25 μm for immunofluorescence. *B,* interaction between Hbxip and Pdx-1 was detected by co-IP assays in INS-1 cells *in vivo*. *C,* direct interaction between recombinant GST-Hbxip and His-Pdx-1 proteins was detected by GST pulldown assays followed by Western blot analysis (*WB*) *in vitro*. *D* and *E,* Pdx-1 is divided into three fragments. GST pulldown assays were performed with GST or GST fusion proteins and His tag fusion proteins containing the indicated amino acid residues of Pdx-1. The proteins were purified from bacteria by glutathione-Sepharose beads. *F,* INS-1 cells were transfected with Pdx-1 siRNA. ChIP assays were conducted 24 h after transfection. *G,* schematic showing the *Ins* mini-enhancer regions with Pdx-1-binding sites or negative control regions without Pdx-1-binding sites. *H,* interaction between Hbxip, Pdx-1, and Neurod1 was detected by co-IP assays in INS-1 cells *in vivo*. *I,* interaction of Pdx-1 with Neurod1 was detected by co-IP assays in INS-1 cells transfected with si-Control or si-Hbxip. *J,* interaction between Hbxip, Pdx-1, and Neurod1 was detected by co-IP assays in the primary β-cells isolated from floxed Hbxip mice. *K,* interaction of Pdx-1 with Neurod1 was detected by co-IP assays in the primary β-cells isolated from floxed Hbxip mice transduced with adeno-LacZ or adeno-Cre. *L,* interaction of Hbxip with Neurod1 was detected by co-IP assays in INS-1 cells transfected with si-Control or si-Pdx-1. *M,* Venn diagram shows the overlapping target genes of HBXIP and Pdx-1. *N, left,* real-time PCR analysis was performed to measure 10 of the 149 candidate target genes in pancreas islets of Hbxip-deficient mice and Ins2-cre (wildtype) mice; *right,* functional assignments for candidate target genes were verified in real-time PCR analysis. Mean ± S.D., *n* = 5/group. Each experiment was repeated at least three times.
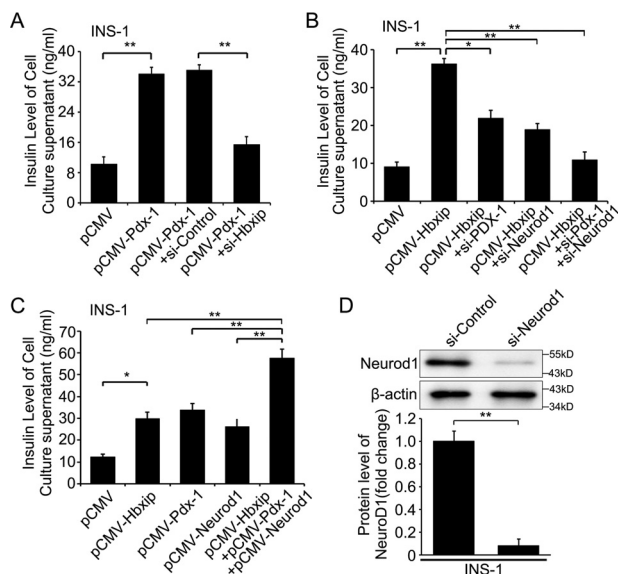
Figure 6. Hbxip increases the insulin expression in pancreatic β-cells through activating Pdx-1 and Neurod1. *A–C*, insulin levels were examined by insulin assay kit in the culture supernatant of INS-1 cells transfected with relative plasmids and siRNAs. *D*, interference efficiency of Neurod1 siRNA was validated by Western blot analysis in INS-1 cells. The protein levels of Neurod1 from three independent experiments were quantified using ImageJ software as shown. Mean ± S.D. Each experiment was repeated at least three times. Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

Neurod1 in INS-1 cells (Fig. 5*I*), suggesting that Hbxip enhanced the interaction between Pdx-1 and Neurod1. This conclusion was further confirmed in the primary β-cells isolated from floxed Hbxip mice according to previous research (Fig. 5, *J* and *K*) (33). Moreover, down-regulation of Pdx-1 was able to attenuate the interaction of Hbxip with Neurod1 (Fig. 5*L*), revealing that Hbxip interacted with Neurod1 through Pdx-1. Together, Hbxip forms a complex with Pdx-1 and Neurod1 in the insulin enhancer in rat pancreatic β-cells.

In addition, we asked whether Hbxip was involved in the modulation of other target genes of Pdx-1. Comparing HBXIP target genes (Table S3) with the data of Pdx-1 ChIP-microarray (34), we found that 18.2 (149/817)/6.9% (149/2170) for Pdx-1/ HBXIP target gene promoters could be occupied by both HBXIP and Pdx-1 in the cells (Fig. 5*M* and Table S4). Furthermore, 10 of the 149 candidate target genes were randomly selected for confirmation by real-time PCR analysis (Fig. 5*N*), indicating that most of the genes in the pancreas islets were decreased in Hbxip-deficient mice relative to those of Ins2-cre mice. Thus, Hbxip is involved in the expression of many other Pdx-1 target genes.

Hbxip increases the insulin expression in pancreatic β-cells through activating Pdx-1 and Neurod1

Finally, we evaluated the effect of Hbxip on insulin production in the culture supernatant of pancreatic β-cells. According to previous studies, high-level glucose is able to enhance the insulin expression in INS-1 and RIN-m5f cells (35, 36). Our data first demonstrated that the knockdown of Hbxip attenuated the glucose-increased insulin levels in the culture supernatant of INS-1 and RIN-m5f cells (data not shown), suggesting that Hbxip contributed to the modulation of insulin mediated by

glucose. Additionally, the knockdown of Hbxip could also attenuate the Pdx-1-enhanced insulin level in the culture supernatant of INS-1 cells (Fig. 6*A*). Moreover, Pdx-1 and/or Neurod1 knockdown markedly abolished the Hbxip-increased insulin level in the culture supernatant of the cells (Fig. 6*B*). Finally, we observed that overexpression of Hbxip, Pdx-1, and Neurod1 or co-expression of the three proteins could up-regulate the insulin level in the culture supernatant of the cells (Fig. 6*C*), supporting that Hbxip was involved in the Pdx-1/Neurod1-mediated up-regulation of insulin. The results were further confirmed in RIN-m5f cells (Fig. S2, *A–C*). The interference efficiencies of Neurod1 siRNAs in INS-1 or RIN-m5f cells and Hbxip and Pdx-1 siRNAs in RIN-m5f cells were validated by Western blot analysis (Fig. 6*D* and Fig. S2, *D–F*). Thus, we conclude that Hbxip increases the insulin expression in pancreatic β-cells through activating Pdx-1 and Neurod1.

Discussion

The peptide hormone insulin is produced in the β-cells of the pancreas and is very crucial in the maintenance of blood glucose homeostasis (37). Our group has reported that HBXIP is able to induce glucose metabolism reprogramming in the development of breast cancer (18, 19). However, the physiological role of HBXIP in glucose metabolism in normal tissues is not well documented. In this study, we investigated the significance of Hbxip in modulation of insulin transcription in animal pancreatic β-cells.

We first found that HBXIP was highly expressed in pancreas islets compared with other parts of the pancreas. Because the insulin from pancreatic β-cells can function in modulating blood glucose levels, we wondered whether HBXIP as a co-activator played a crucial role in transcriptional regulation of insulin, the key gene for maintaining normoglycemia. Then, we developed the pancreatic β-cell–specific Hbxip-knockout mice. As expected, we found that pancreatic Hbxip deletion resulted in the higher blood glucose and attenuated glucose tolerance in fasting conditions in mice. Thus, we supposed that Hbxip might be involved in the regulation of insulin. Then, we showed that Hbxip was able to increase the production of insulin in the supernatant of INS-1 cells. Moreover, Hbxip was able to up-regulate insulin expression in INS-1 cells. Next, we try to identify the mechanism by which Hbxip increases the expression level of insulin. As the E2A3/4 mini-enhancer is able to give the characteristic activity of the full promoter (10), the *Ins* mini-enhancer cloning in the pGL3-Basic plasmid can be used to identify the relationship between Hbxip and the insulin promoter. It has been reported that Pdx-1 as a key transcription factor can up-regulate insulin at the transcription level (38, 39). Because Hbxip acts as a co-activator of transcription factors, we speculated that Hbxip might activate Pdx-1 to stimulate the insulin enhancer. As expected, we found that Hbxip as a co-activator of transcription factor Pdx-1 increased insulin enhancer activity through direct interaction with Pdx-1 in pancreatic β-cells. Moreover, growing evidence has revealed that Neurod1 is crucial for the activity of Pdx-1 by forming a dimer with Pdx-1 during the transcription of insulin (8). Interestingly, we demonstrated that Hbxip, Neurod1, and Pdx-1 formed a complex to activate the insulin enhancer in pancreatic β-cells. In addition,
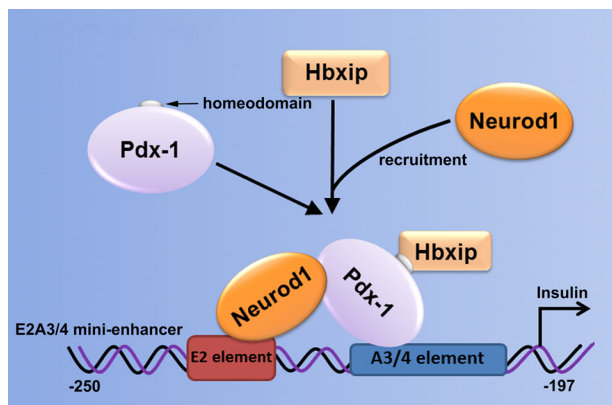
Figure 7. Model shows that Hbxip contributes to the modulation of insulin transcription. Hbxip acting as a co-activator promotes insulin transcription through binding to the mini-enhancer of insulin and activating transcription factor Pdx-1. Hbxip co-activates Pdx-1 through recruiting Neurod1 to Pdx-1 forming a transcription factor complex of Hbxip/Pdx-1/Neurod1. Thus, Hbxip increases the insulin expression by increasing levels of the Pdx-1/Neurod1 complex in animal pancreatic β-cells.

our data revealed that Hbxip was also involved in the expression of many other Pdx-1 target genes.

Interestingly, although Hbxip functioned as a co-activator in the nuclei of β-cells, it was still observed both in the nuclei and cytoplasm of islet cells under physiological conditions as shown in Fig. 1. This finding may indicate other functions of Hbxip in pancreas islets. Finally, our data confirmed that Hbxip increased the insulin expression in pancreatic β-cells by activating Pdx-1 and Neurod1.

It has been reported that the onset of most type 1 diabetes is related to insulin production deficiency and injury of islet tissues (40, 41). Type 1 diabetes results from a chronic autoimmune process continuing for years after presentation (42). However, the significance of Hbxip in the onset of type 1 diabetes remains unclear.

In summary, we present a model that Hbxip contributes to the modulation of insulin transcription in Fig. 7. In this study, we show that Hbxip co-activates transcription factor Pdx-1 by enhancing the interaction between Pdx-1 and Neurod1 to promote the insulin transcription in pancreatic β-cells. Thus, our finding provides the insight into the mechanism by which Hbxip physiologically modulates the insulin expression in animal pancreatic β-cells.

Experimental procedures

Construction of pancreatic β-cell–specific Hbxip-knockout mice

The Hbxip conditional knockout mice (Hbxip-deficient mice) were generated using the Cre-loxP approach (20). LoxP was inserted into the flank regions of Hbxip exon2. The pancreas conditional knockout mice were generated by crossing floxed Hbxip mice with the Ins2-cre transgenic mice, B6.Cg-Tg(Ins2-cre)25Mgn/J (commonly called RIP-Cre), in which the Cre recombinase was expressed selectively in pancreatic β-cells (43, 44). The Ins2-cre transgenic mice were purchased from Model Animal Research Center of Nanjing University (n000117). The genotypes of filial mice were identified by PCR analysis using primers upstream and downstream of the loxP in the intron of Hbxip gene. Analysis of the phenotype was conducted in Hbxip-deficient mice, and Ins2-cre littermates were used as wildtype controls. The 7-week-old male Hbxip-deficient mice and Ins2-cre mice were used in this study. The mouse housing environment includes a 12:12-h light/dark cycle, constant room temperature (22–25 °C), and free access to water and diet. All procedures involving animals were performed according to the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were performed according to the institutional ethical guidelines for animal experiments. All experiments were approved by the Institute Research Ethics Committee at Nankai University. The primers used for the loxP and Cre identification were listed in Table S1.

Cell culture and treatment

Rat pancreatic β-cell lines, such as INS-1 and RIN-m5f (commonly called rat pancreatic insulinoma β-cells), were cultured in RPMI 1640 medium (Gibco) containing 11.2 mM glucose according to previous research (28, 36). Human kidney epithelial (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (Gibco). All cell lines were supplemented with heat-inactivated 10% fetal bovine serum (Gibco), 100 units/ml penicillin, and 100 mg/ml streptomycin and grown at 5% CO_2 and 37 °C. Cells were collected and seeded in 6- or 24-well plates for 24 h and then transfected with plasmids or siRNAs. All transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

HE and IHC staining

The tissues of human and mice were fixed in formalin and were dehydrated and embedded in paraffin for HE and IHC staining as described previously (22, 45). Some of the results were quantified using ImageJ software. The tissue microarray (No. AM00A01) was purchased from Aomeibio Co. (Xian, China). Written consent approving the use of tissue samples for research purposes was obtained from patients. Informed consent for study participation was also obtained from each patient. Detailed information of normal tissues is shown in Table S2. The study protocol was approved by the Institute Research Ethics Committee at Nankai University. All experiments were performed strictly in accordance with relevant guidelines and regulations.

Western blot analysis

Western blot analysis was carried out with the standard protocols (45). The primary antibodies used were rabbit anti-Pdx-1 (Proteintech Group, 20989-1-AP), mouse anti-Neurod1 (Wuhan Boster Biological Technology Ltd., BM3336), rabbit anti-Hbxip (Santa Cruz Biotechnology, Santa Cruz, CA, sc-373980), rabbit anti-insulin (Proteintech Group, 15848-1-AP), mouse anti-His tag (Proteintech Group, 66005-1-Ig), and mouse anti-β-actin (Sigma, A1978). For relative experiments, protein was prepared from pure β-cells, as well as non-β-cells, obtained by flow cytometry according to previous work (24, 46). Some of the results were quantified using ImageJ software.

Hbxip participates in the transcription of insulin

Plasmid construction and small interfering RNA

pGL3-Basic vector was from Promega (Madison, WI), and RNA (siRNA), pCMV-tag2B, adeno-LacZ control vector, pCMV-Hbxip, and pCMV-HBXIP were kept in our laboratory. The complete human PDX-1 cDNA (GenBank^{TM} accession number NM_000209.3), rat Pdx-1 cDNA (GenBank^{TM} accession number NM_022852.3), or Neurod1 cDNA (GenBank^{TM} accession number NM_019218.2) was subcloned into the pCMV-tag2B vector to generate the pCMV-PDX-1, pCMV-Pdx-1, or pCMV-Neurod1 construct, respectively. The E2A3/4 mini-enhancer of the rat insulin promoter region (bp −247 to −197) with a wildtype or mutant Pdx-1-binding site (10) was synthesized from the Riobio Co. (Guangzhou, China), and subcloned into the pGL3-Basic vector to generate the pGL3-Ins or pGL3-Ins-mut construct. The 2′-O-methyl interfering RNAs directly against HBXIP or Hbxip, Pdx-1, Neurod1, and the control siRNAs were purchased from the Riobio Co. The sequences of siRNAs for HBXIP, Hbxip, and Neurod1 were described previously (47, 48). The sequences of relative primers and siRNA for Pdx-1 were described in Table S1.

Islet isolation from mouse pancreas

The pancreas islets were isolated from 7-week-old male Hbxip-deficient mice, Ins2-cre littermates, and floxed Hbxip mice according to a previous report (49).

Total RNA isolation, reverse transcription-PCR (RT-PCR), and real-time PCR

Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized with the PrimeScript reverse transcriptase kit (TaKaRa Bio, Shiga, Japan). RT-PCR and real-time PCR were performed as described previously (50). The primers used for relative genes cDNA were described in Table S1.

Animal treatments

For the HFD study, 5-week-old mice were subjected to NCD or 60% HFD (Research Diets, Inc.) for 25 weeks. For IPGTT or IVGTT, overnight-fasted mice were injected intraperitoneally or intravenously with glucose (2 g/kg body weight). Blood glucose levels and insulin levels were measured from tail vein blood collected at the designated times using Bayer Brand glucometer (Bayer Health Care, Mishawaka, IN) and insulin assay kit (Joyee Biotechnics, Shanghai, China). Data were expressed in blood glucose levels (mg/dl) or plasma insulin levels (ng/ml). For intraperitoneal-insulin tolerance tests, mice were fasted for 6 h, and basal blood samples were taken, followed by intraperitoneal injection of insulin (0.4 units/kg for NCD mice and 0.6 units/kg for HFD mice). All the studies were performed according to the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were performed according to the institutional ethical guidelines for animal experiments. All experiments were approved by the Institute Research Ethics Committee at Nankai University.

Measurement of insulin secretory response from isolated islets

For static GSIS assays, mouse islets were incubated for 2 h in low glucose media at 37 °C, 5% CO_2 and then incubated for 60 or 75 min with 2.8 or 16.7 mM glucose in the same conditions. The experiments were performed as described previously (51, 52).

Insulin level of cell culture supernatant

The cell culture supernatant of INS-1 and RIN-m5f cells transfected with plasmids or siRNAs was collected 48 h after transfection and measured by insulin assay kit (Joyee Biotechnics, Shanghai, China).

Luciferase reporter gene assays

Adherent cells were seeded into 24-well plates and co-transfected with pGL3-Ins, pGL3-Basic, or pGL3-Ins-mut and the pRL-TK plasmid (Promega). Cell extracts were harvested after 36 h and lysed using lysis buffer (Promega). Luciferase reporter gene assays were implemented using the Dual-Luciferase® reporter gene assay system (Promega) according to the manufacturer's instructions. All experiments were performed at least three times.

Confocal microscopy

Confocal microscopy was performed as described previously (53), and the cells were incubated with RPMI 1640 medium containing 15 mM glucose and transfected with relative plasmids. Cells were observed under the confocal microscopy (Leica TCS SP5).

Co-immunoprecipitation assays

The co-IP assays were performed according to previously published articles with INS-1 cells and primary β-cells isolated from floxed Hbxip mice (54). The cells were harvested and lysed in a lysis buffer (50 mmol/liter Tris-HCl, pH 8.0, 100 mmol/liter NaCl, 50 mmol/liter sodium fluoride, 1% Nonidet P-40, 1 mmol/liter dithiothreitol, 1 mmol/liter Na_3VO_4, 1 mmol/liter Microcystin-LR, 1 mmol/liter phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml aprotinin). The lysates were incubated with antibodies (rabbit anti-Hbxip or IgG)/protein G-conjugated agarose beads (Millipore). The precipitates were washed six times with ice-cold lysis buffer, resuspended in PBS, followed by Western blot analysis. For Western blot analysis, the following were used: Pdx-1, primary antibody is rabbit anti-Pdx-1 (Proteintech Group, 20989-1-AP); secondary antibody is IPKine HRP mouse anti-rabbit IgG light chain (Abbkine, A25022); Neurod1, primary antibody is mouse anti-Neurod1 (Wuhan Boster Biological Technology Ltd., BM3336); secondary antibody is IPKine HRP goat anti-mouse IgG light chain (Abbkine, A25012); Hbxip, primary antibody is rabbit anti-Hbxip (Santa Cruz Biotechnology, Santa Cruz, CA, sc-373980); and secondary antibody is IPKine HRP goat anti-rabbit IgG heavy chain (Abbkine, A25222).

GST pulldown assays

The cDNA of Hbxip was cloned into the pGEX-4T1 expression vector; the cDNA of Pdx-1 was divided into three frag-

ments according to a previous report (10), and the full-length or fragments of Pdx-1 were cloned into the pET-28a expression vector. The detailed procedure was performed according to published protocols (55).

ChIP assays and the overlap of HBXIP and Pdx-1 binding

The ChIP assays were performed using an EpiQuik^{TM} chromatin immunoprecipitation kit from Epigentek Group Inc. (Brooklyn, NY) and according to the published methods (16, 56). Protein/DNA complexes were immunoprecipitated with anti-Hbxip antibody, with anti-RNA polymerase II as a positive control antibody, and with mouse IgG as a negative control antibody. Amplification of soluble chromatin prior to immunoprecipitation was used as an input control. DNA from these samples was then subjected to PCR analysis. Primer sets for the *Ins* mini-enhancer and negative control regions are shown in Table S1. Experiments were repeated three times. According to previous report (34), 817 Pdx-1 target genes were obtained from the datasets of ChIPseq for Pdx-1, which was produced by the Beta Cell Biology Consortium (Mouse PromoterChip BCBC-5B, see BCBC website). The ChIP-DNA Selection and Ligation (ChIP-DSL) and data analysis of HBXIP-bound genes were performed by CapitalBio Corp. according to protocol from Aviva Systems Biology. Experiments were repeated three times, and the results were analyzed using MAS (http://mas.capitalbiotech.com/mas3/)[4] with a p value cutoff of 1.0×10^6 for promoter identification. The candidate target genes of HBXIP (2170) are shown in Table S3. According to the gene name, the Pdx-1 target genes were used to merge with the HBXIP-bound genes for overlapping. The datasets for 149 merged genes of Pdx-1 with HBXIP are provided in Table S4.

Isolation of primary β-cells

The primary β-cells were isolated from 8-week-old male floxed Hbxip mice according to a previous report (33).

Statistical analysis

Each experiment was repeated at least three times. Statistical significance, assessed by comparing mean values ± S.D., was assumed for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). All statistical analysis was performed by Student's *t* test or analysis of variance in Excel (Microsoft).

Author contributions—H. L. performed experiments, analyzed data, and wrote the manuscript; Z. W., Y. L., and R. F. performed experiments and provided technical assistance; H. W. and H. S. generated tools and contributed to the preparation of the figures; X. Z. designed part of the study; L. Y. and W. Z. designed the study and wrote the paper.

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