Islet α-, β-, and δ-Cell Development Is Controlled by the Ldb1 Coregulator, Acting Primarily With the Islet-1 Transcription Factor

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Ldb1 and Ldb2 are coregulators that mediate Lin11-Isl1-Mec3 (LIM)–homeodomain (HD) and LIM-only transcription factor–driven gene regulation. Although both *Ldb1* and *Ldb2* mRNA were produced in the developing and adult pancreas, immunohistochemical analysis illustrated a broad Ldb1 protein expression pattern during early pancreatogenesis, which subsequently became enriched in islet and ductal cells perinatally. The islet-enriched pattern of Ldb1 was similar to pan-endocrine cell–expressed Islet-1 (Isl1), which was demonstrated in this study to be the primary LIM-HD transcription factor in developing and adult islet cells. Endocrine cell–specific removal of Ldb1 during mouse development resulted in a severe reduction of hormone$^+$ cell numbers (i.e., α, β, and δ) and overt postnatal hyperglycemia, reminiscent of the phenotype described for the *Isl1* conditional mutant. In contrast, neither endocrine cell development nor function was affected in the pancreas of *Ldb2*$^{-/-}$ mice. Gene expression and chromatin immunoprecipitation (ChIP) analyses demonstrated that many important Isl1-activated genes were coregulated by Ldb1, including *MafA*, *Arx*, *insulin*, and *Glp1r*. However, some genes (i.e., *Hb9* and *Glut2*) only appeared to be impacted by Ldb1 during development. These findings establish Ldb1 as a critical transcriptional coregulator during islet α-, β-, and δ-cell development through Isl1-dependent and potentially Isl1-independent control. ***Diabetes*** **62:875–886, 2013**

The vertebrate pancreas is composed of acinar, endocrine, and ductal cells critical for maintaining nutritional homeostasis. Although acinar and ductal cells secrete and transport enzymes important for food digestion, endocrine-derived α, β, δ, and pancreatic polypeptide (PP) cells of the islets of Langerhans produce hormones essential for regulating glucose homeostasis. For example, insulin secreted from β-cells is critical for glucose uptake in peripheral tissues, whereas glucagon released from α-cells acts in a counterregulatory manner to promote gluconeogenesis and glycogenolysis (1).

Pancreatic organogenesis begins at embryonic day (E) 9.5 when dorsal and ventral buds form from a regionalized domain of the foregut endoderm. This process is influenced by both extrinsic signals from the adjacent mesenchyme and intrinsic lineage-specific transcription factors like Ptf1a and Pdx1 (2–5). Several days later, endocrine cells arise from neurogenin 3 (Ngn3) expressing endocrine progenitors that reside within the ductal epithelium (6–9). These cells delaminate and aggregate into hormone$^+$ endocrine clusters, which then proliferate between E13 and 18.5 (2). Differentiation of endocrine lineages occurs in part through cell type–specific expression of several islet-enriched transcription factors, including Arx, Isl1, MafB, Nkx6.1, Nkx2.2, Pax4, and Pax6 (10). The levels of Pdx1, MafA, and Nkx6.1 (11–13) are enhanced in mature islet β-cells, whereas Arx and MafB are restricted to α-cells (14–17).

Interestingly, Isl1 is produced in both the developing pancreatic epithelium and surrounding lateral and dorsal mesenchyme at ~E9.5, with expression then becoming restricted to all postnatal islet cells (18,19). Although *Isl1*$^{-/-}$ mice die embryonically from severe heart defects (i.e., ~E10.5), impaired formation of the dorsal pancreatic epithelium and mesenchyme was observed (19). A conditional deletion strategy was subsequently used to study the function of Isl1 in endocrine progenitors by crossing floxed (F) *Isl1* mice with transgenic *Pdx1*^{Late}-Cre mice (20,21), which catalyzed recombination in pancreatic epithelial cells by E13.5. *Isl1*-deficient mice became overtly hyperglycemic due to greatly reduced numbers of α-, β-, and δ-cells, a phenotype caused by effects on proliferation, apoptosis, and maturation due (in part) to actions on *MafA* and *Arx* transcription (22,23).

LIM domain [derived from Lin11-Isl1-Mec3 (24)] factors like Isl1 (LIM-homeodomain [HD]) and related LIM-only (Lmo) proteins act through binding with the LIM-domain–binding coregulators Ldb1 (also called CLIM2, Nuclear LIM Interactor, and Chip) and/or Ldb2 (25–27). Strikingly, there are ~250 known coregulators (www.nursa.org), yet only a few have been associated with pancreatic development or adult islet cell function [e.g., cAMP-responsive element–binding protein (CBP)/p300, p300/CBP–associated factor, Pdx-1 COOH terminus–interacting factor 1, Set7/9, and Bridge-1 (28–34)]. In this study, we analyzed how Ldb1 and/or Ldb2 influence pancreatic endocrine cell development. *Ldb1* and *Ldb2* mRNA was expressed in developing pancreatic and adult islet cells, with *Ldb1* more abundant. In addition, Ldb1 protein was widely distributed in the early pancreatic epithelium and surrounding mesenchyme, eventually becoming enriched in endocrine and ductal cells. Ldb1 removal in developing mouse Pax6$^+$ endocrine cells reduced insulin$^+$ (i.e., β), glucagon$^+$ (α), and somatostatin$^+$ (δ) cell formation in a manner similar to

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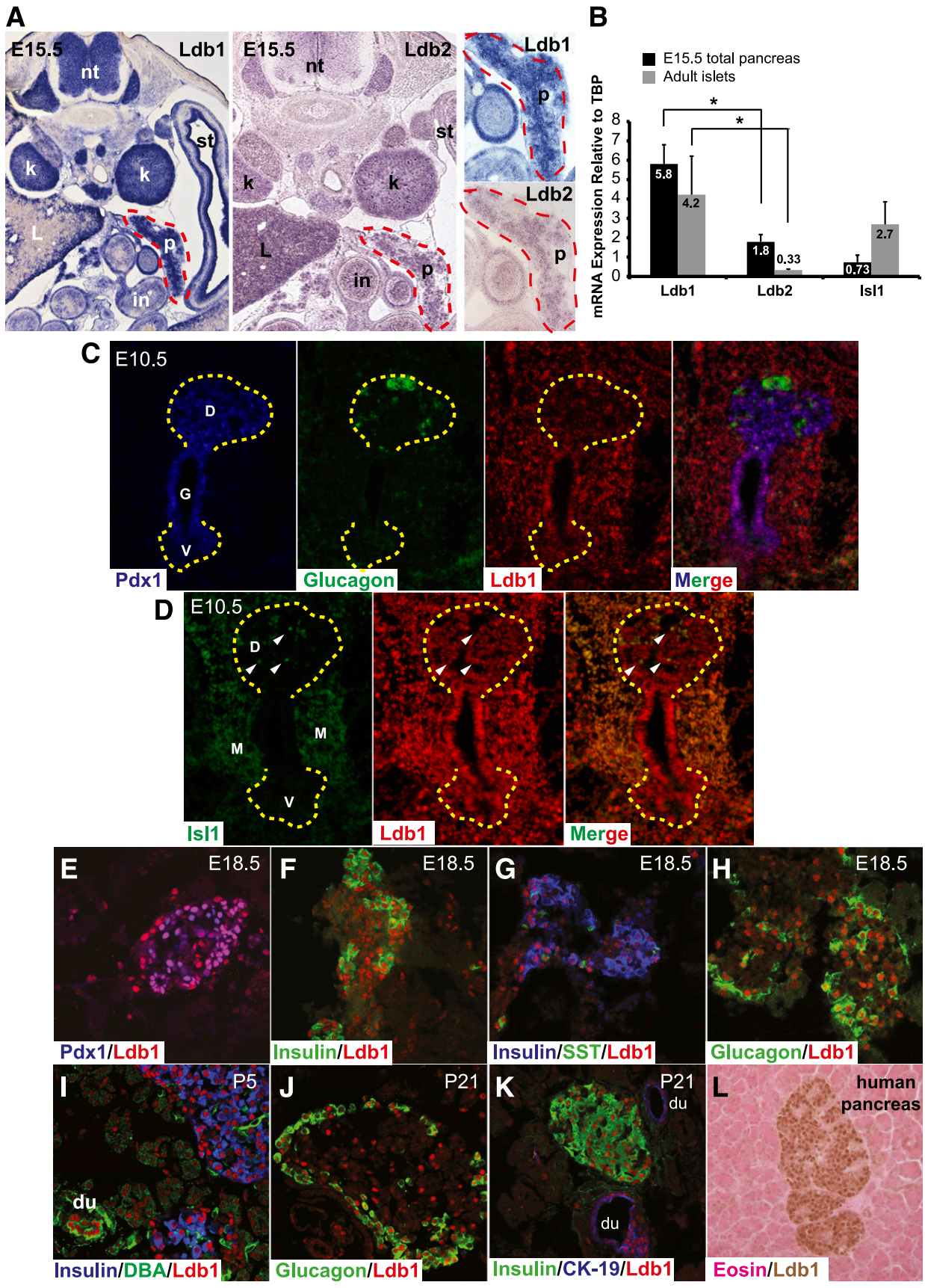

FIG. 1. Ldb1 is enriched in islet and ductal cells. *A*: *Ldb1* (*left*) and *Ldb2* (*middle*) mRNA expression was visualized by RNA ISH under identical conditions in E15.5 tissue. A higher-magnification view of pancreatic *Ldb1* and *Ldb2* expression is shown on the *right*. *B*: qPCR was performed to measure *Ldb1*, *Ldb2*, and *Isl1* mRNA levels in E15.5 total pancreas (black bars) and 3-month-old isolated islets (gray bars). Expression levels are displayed relative to TATA-binding protein (TBP), which is set as onefold. Error bars represent ± SEM (n = 5). *Ldb1* mRNA is significantly more

Pdx1^{Late}*-Cre*;*Isl1*$^{F/F}$ mice, perhaps not surprisingly considering the relative abundance of *Isl1* mRNA levels to other pancreatic LIM-HD–expressed genes. In contrast, endocrine cell development in *Ldb2*$^{-/-}$ mutants was unaffected. Gene expression and chromatin immunoprecipitation (ChIP) analyses showed that Ldb1 control was primarily linked to Isl1 activation (22,23). However, distinct and novel Ldb1 regulatory actions were also found during development, suggesting essential contributions of other LIM-HD and/or Lmo factors in islet cell formation and function.

RESEARCH DESIGN AND METHODS

Animals. *Ldb1*$^{F/F}$ (35), *Ldb2*$^{-/-}$ [Mouse Genome Informatics, Lexicon Genetics (36)], *Pdx1*^{Late}*-Cre*;*Isl1*$^{F/F}$ (22), and *Pax6-Cre* (also called *Le-Cre*) (37) mice have been described previously; the lines were maintained on a mixed (B6) background. Cre was first visualized at ~E11.5 in *Pdx1*^{Late}*-Cre* endocrine cells (21), at least 24 h after other transgenic ~4.5-kb promoter-driven *Pdx1-Cre* lines (38). *Pax6-Cre*;*Ldb1*$^{F/F}$ and control littermate mice (*Ldb1*$^{F/+}$, *Ldb1*$^{F/F}$, and *Pax6-Cre*;*Ldb1*$^{F/+}$ genotypes) were generated by mating *Pax6-Cre*;*Ldb1*$^{F/+}$ to *Ldb1*$^{F/F}$ mice. The morning of vaginal plug discovery was considered E0.5. The Vanderbilt University and Children's Hospital of Philadelphia Institutional Use and Care Committees approved all of the animal experiments.

Fasting blood glucose measurements. Postnatal (P) day 10 to P26 mice were fasted for 6 h, and blood glucose was measured from the tail vein using a BD-Logic glucometer (Nova Biomedical, Waltham, MA) and Nova Max test strips (Nova Diabetes Care). Some *Pax6-Cre*;*Ldb1*$^{F/F}$ animals exceeded the 600 mg/dL limit of the meter, but were still referred to as 600 mg/dL. All numerical data are presented ± SEM. Significance was determined after performing an unpaired *t* test, for which $P < 0.05$.

RNA isolation, cDNA synthesis, and quantitative real-time PCR. Control, *Ldb1*-mutant, and *Isl1*-mutant E15.5–18.5 pancreata were quickly excised and stored at −20°C in RNA-Later Ice (#AM7030; Ambion/Life Technologies, Carlsbad, CA). RNA was extracted using the RNeasy Mini kit (#74104; Qiagen, Valencia, CA) after homogenizing the tissue with a T8 Ultra-Turrax disperser (IKA Works, Wilmington, NC). cDNA was prepared from this RNA using oligo (dT) and the iScript cDNA synthesis kit (#170-8891; Bio-Rad, Hercules, CA). Quantitative real-time PCR (qPCR) reactions were performed in triplicate with reference gene normalization using the SYBR Green PCR master mix (Roche, Indianapolis, IN) in a LightCycler 480 II (Roche). See Supplementary Table 1 for primer sequences.

Immunohistochemical and in situ RNA hybridization analyses. Staged embryonic and postnatal littermate-matched control, *Pax6-Cre*;*Ldb1*$^{F/F}$, and *Pdx1*^{Late}*-Cre*;*Isl1*$^{F/F}$ pancreatic tissues were fixed in 4% paraformaldehyde and embedded in paraffin or Optimal Cutting Temperature (Tissue-Tek). Sections were cut to 6–12 μm and blocked with 5% normal donkey serum in 1% BSA/1× PBS and then incubated with primary antibodies overnight at 4°C (see Supplementary Table 2 for antibody type, dilution, and specific staining conditions). Cy2-, Cy3-, or Cy5-conjugated donkey anti–guinea pig, anti-mouse, anti-goat, or anti-rabbit IgG secondary antibodies (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) were used for detection. Lmo4, Glp1r, some insulin, and Ldb1 primary signals were visualized using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Slides were imaged by confocal microscopy using a Zeiss LSM510 or by fluorescent/brightfield microscopy using a Zeiss Axioimager M2 (Zeiss) and the images processed by LSM (Zeiss) or ImageJ (National Institutes of Health) software.

In situ hybridization (ISH) analysis was performed on E15.5 embryos fixed in 4% paraformaldehyde followed by overnight saturation in 30% sucrose solution. Embryos were embedded in Optimal Cutting Temperature (Tissue-Tek) and sectioned to 10 μm. Prehybridization was performed in a solution of 50% formamide, 5× SSC (pH 4.5), 50 μg/mL yeast tRNA (Sigma-Aldrich), 1% SDS, and 50 μg/mL heparin (Sigma-Aldrich) at 55°C for 1 to 2 h, and hybridization was at 70°C overnight using *Ldb1* (600 base pairs) and *Ldb2* (578 base pairs) coding region probes at 300–400 ng/mL. Slides were washed in a solution of 2% blocking reagent (Roche), 10% heat-inactivated sheep serum, 0.1% Tween-20, and 1× maleic acid buffer for 1 h at room temperature. Anti-digoxigenin antibody (1:2,000; Roche) was diluted in blocking solution and incubated overnight at 4°C. Slides were washed first in 1× maleic acid buffer/0.1% Tween and then with 0.1% Tween before BM Purple (Roche) was added. After the signal developed, slides were rinsed in PBS and mounted with Fluoroshield (Axell).

ChIP. βTC-3 and αTC-6 monolayer cells (~4×10^6 cells) were cross-linked with 1% formaldehyde and chromatin fragmentation performed as described previously (22). Chromatin was precleared with protein G-Sepharose (#101242; Invitrogen/Life Technologies, Carlsbad, CA) and then incubated with anti-Ldb1 (sc-11198X; Santa Cruz Biotechnology), anti-Isl1 (DSHB, 39.4D5), species-matched preimmune IgG (Santa Cruz Biotechnology), or without antibody. Bound complexes were precipitated with BSA- and salmon sperm DNA-blocked protein G-Sepharose. The eluted and immunoprecipitated DNA (1:20) was used in a PCR reaction with Taq polymerase Hotstart Mastermix (5 Prime, Gaithersburg, MD) and 12.5 pmol of each primer (see Supplementary Table 1). PCR parameters were: 95°C for 2 min (1 cycle), 95°C for 30 s, 59 to 60°C for 30 s, and 72°C for 30 s (29 to 30 cycles). Reaction products were separated on 1.5–2.0% agarose gels using 1× Tris–acetate–ethylenediaminetetraacetic acid buffer and visualized with ethidium bromide. Experiments were performed with at least three independently isolated chromatin preparations.

Coimmunoprecipitation and immunoblotting. βTC-3 nuclear extract was prepared in the presence of protease inhibitor cocktail (Sigma-Aldrich) as described previously (39). Covalent antibody-bound anti-Ldb1–, anti-Isl1–, or control IgG Dynabeads (Invitrogen) were incubated with 400 μg of extract protein for 3 h at 4°C. The beads were then washed five times with PBS and bound proteins eluted with RIPA buffer (50 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) at 37°C. The eluted material was separated by 10% SDS-PAGE (NuPAGE; Invitrogen) and electrophoretically transferred to polyvinylidene difluoride membrane. The membrane was blocked in PBS/Tween supplemented with 5% nonfat dry milk followed by incubation overnight with anti-Ldb1 (1:2,000; see Supplementary Table 2 for additional antibody sources), anti-Isl1 (1:2,000), anti-Pdx1 (1:20,000), anti-Pax6 (1:1,000), anti-NeuroD1 (1:3,000; 3181-1; Epitomics), anti-Hnf1α (1:2,000, sc-6548X; Santa Cruz Biotechnology), and anti-MafA (1:2,000, A300 BL-1225; Bethyl Laboratories). The washed membrane was incubated with horseradish peroxidase-conjugated secondary antibody followed by detection using Western-Lightning Plus-ECL (PerkinElmer, Waltham, MA).

Transient transfection and reporter gene assays. The wild-type pFox-mouse *MafA* region 3-luciferase plasmid (40) was cotransfected using Lipofectamine reagent (Invitrogen) into βTC-3 cells with cytomegalovirus (CMV) enhancer-driven Ldb1 dominant-negative acting Ldb1ΔN and the Renilla phRL-TK internal control. Ldb1ΔN spans amino acids 200–375 and contains the LIM interaction domain, but not the dimerization domain (41). Lysates were prepared 48 h posttransfection and analyzed using the Dual Luciferase assay according to the manufacturer's protocol (Promega, Madison, WI). Each transfection was performed in triplicate on at least three independent occasions; firefly luciferase activity levels were normalized to Renilla.

RESULTS

Ldb1 is broadly expressed in the early pancreatic epithelium and surrounding mesenchyme, then becomes enriched in islet and ductal cells perinatally. *Ldb1* and *Ldb2* mRNA expression was observed in the pancreas, neural tube, and kidney by ISH (Fig. 1*A*). Notably, *Ldb1* levels were ~3.2-fold higher than *Ldb2* in E15.5 pancreata by qPCR analysis and 12.7-fold in adult islets (Fig. 1*B*). *Ldb1* mRNA was also much more abundant than *Isl1* in the E15.5 pancreas (Fig. 1*B*), presumably reflecting a broader cell distribution than endocrine cell–specific Isl1 (18,19).

Immunostaining analysis was next performed to characterize temporal and spatial Ldb1 protein expression in the developing and adult pancreas. At E10.5, Ldb1 was widely

abundant than *Ldb2* in E15.5 and adult samples. *C–K*: Ldb1, Pdx1, Isl1, hormone (insulin, glucagon, and somatostatin), and ductal (DBA, CK-19) markers were visualized at E10.5, E18.5, P5, and P21 by coimmunofluorescence. Yellow dashed lines mark dorsal and ventral pancreas domains in *C* and *D*. Notably, only a few of the pancreatic Ldb1$^+$ cells in *D* are copositive for Isl1 at this stage (some marked by white arrowheads). *L*: Immunohistochemical analysis illustrates enriched Ldb1 protein (brown) expression in adult human islet cells; the sample is eosin (pink) counterstained. *$P < 0.05$. D, dorsal pancreas; du, duct; G, gut tube; in, intestine; k, kidney; L, liver; M, mesenchyme; nt, neural tube; P, pancreas (outlined with red dashed line); st, stomach; V, ventral pancreas. (A high-quality digital representation of this figure is available in the online issue.)

produced in early Pdx1$^+$ pancreas-specified endoderm, mesenchyme, and early glucagon$^+$ cell population (Fig. 1*C*). Isl1 expression was also detected in Ldb1$^+$ cells in lateral and dorsal mesenchyme (Fig. 1*D*) and in a small subset of Ldb1$^+$ cells in the pancreatic epithelium (Fig. 1*D*) (19). Later in pancreatic development, Ldb1 expression became more enriched in insulin$^+$/Pdx1$^+$, glucagon$^+$, somatostatin$^+$, and pancreatic polypeptide$^+$ cells than the surrounding acinar cells (Fig. 1*E–H* and data not shown). Islet cell–enriched Ldb1 expression persisted postnatally and was found in all hormone$^+$ subtypes, overlapping with Isl1 expression (Fig. 1*I–K*) (18). In addition, Ldb1 was present in cytokeratin-19$^+$ (CK-19) and *Dolichos biflorus* agglutinin (DBA)$^+$ ductal cells, which lack Isl1 (Fig. 1*I* and *K*) (18). The Ldb1 protein was also enriched in human islet cells (Fig. 1*L*), consistent with recent findings examining both *Ldb1* and *Isl1* mRNA expression in this context (42).

Isl1 is the most abundantly expressed LIM-HD family member in the developing and adult pancreas. Ldb1 and Ldb2 interact with the LIM domains of LIM-HD and Lmo proteins (43). Significantly, these obligate coregulators cannot function in the absence of an associated LIM-factor because they lack the capacity to independently bind *cis*-element DNA, transactivate, or remodel chromatin (43). Expression of all mouse LIM-HD and known Ldb1-interacting Lmo genes was measured in E15.5 pancreata and isolated 3 month-old islets by qPCR to evaluate their abundance. *Lmo2* and *Lmo4* were highly expressed relative to *Isl1* at E15.5, but were reduced in adult islets (Fig. 2*A*). Immunohistochemical analysis showed that Lmo4 was present in E18.5 and adult acinar and ductal cell nuclei, but not endocrine cells (Fig. 2*B* and *C*). As a consequence, the relatively small islet *Lmo4* mRNA signal presumably represents acinar and ductal cell sample contamination. Notably, this distribution pattern was quite distinct from Isl1 (18,19), suggesting a role for Ldb1/2 in Lmo4 gene regulation in the exocrine pancreas. *Isl1* was the principal LIM-HD mRNA detected in E15.5 and adult islet pancreatic samples, with much lower levels of *Lhx1* in adult (Fig. 2*A*). These observations paralleled human mRNA data demonstrating acinar cell enrichment of *Lmo4* and *Isl1* in islets (42). Taken together, these data suggest an important transcriptional relationship among Isl1, Ldb1, and/or Ldb2 in regulating endocrine cell development in the mouse pancreas.

Endocrine cell deletion of Ldb1, and not Ldb2, led to reduced hormone production, postnatal islet cell loss, and hyperglycemia. Our studies next focused on determining the significance of Ldb1 and Ldb2 to endocrine hormone$^+$ cell development in vivo. Notably, *Ldb1*$^{-/-}$ mice fail to develop past ~E8.5 due to defects in heart formation, foregut indentation, and anterior–posterior axis patterning (44), whereas *Ldb2*$^{-/-}$ animals appear overtly normal [Mouse Genome Informatics, Lexicon Genetics (36)]. To circumvent embryonic lethality, *Pax6-Cre* mice (37) were crossed to *Ldb1*$^{F/F}$ mice (35) to specifically remove this coregulator from developing endocrine cells. Immunostaining analyses showed that a majority of Ldb1 protein was selectively removed from hormone$^+$ cells of *Pax6-Cre;Ldb1*$^{F/F}$ animals by E15.5 and absent from E18.5 endocrine cells, with expression remaining in surrounding exocrine cells (Supplementary Fig. 1).

Pax6-Cre;Ldb1$^{F/F}$ mice were born at the predicted Mendelian ratio, with no overt change in body length or weight (data not shown). Both male and female Ldb1-deficient mice had elevated fasting blood glucose levels by P10 that worsened with age (Fig. 3*A*). Notably, a detectible loss of insulin$^+$ islet cells was visible in P6 *Pax6-Cre;Ldb1*$^{F/F}$ mice despite their mild hyperglycemic phenotype, with virtually no islets remaining in the 3–5-week-old pancreata (Fig. 3 and data not shown). All mutant mice were killed by P35 because of the severe hyperglycemia.

A significant reduction in *insulin*, *glucagon*, and *somatostatin* mRNA levels was observed in E18.5 *Ldb1* mutant pancreata, whereas there was little to no effect on *PP* or *ghrelin* expression (Fig. 3*F*). Immunohistochemical analysis of hormone protein expression revealed a similar trend, as insulin$^+$, glucagon$^+$, and somatostatin$^+$ cell numbers were compromised and amylase$^+$, pancreatic polypeptide$^+$, and ghrelin$^+$ cells unchanged in *Pax6-Cre;Ldb1*$^{F/F}$ pancreata (Fig. 3*I* and *J* and data not shown). Notably, these cell types were impacted in an analogous manner in *Pdx1*^{Late}*-Cre;Isl1*$^{F/F}$ mice, which results in postnatal endocrine cell apoptosis in both circumstances (22 and data not shown). In contrast, there was no effect on hormone$^+$ cell development or glucose homeostasis in *Ldb2*$^{-/-}$ mice (Supplementary Fig. 2 and data not shown), despite a broad and overlapping expression pattern with Ldb1 in the developing pancreas (Fig. 1*A*). Collectively, these data demonstrated that Ldb1 was the principal coregulator of LIM-HD and Lmo transcription factor activity in the pancreas. Moreover, as the impact of Ldb1 deficiency on endocrine cell formation and function was comparable to that observed in *Pdx1*^{Late}*-Cre;Isl1*$^{F/F}$ mice (22), these data strongly supported a functional link between Ldb1 and Isl1 in islet α-, β-, and δ-cell development.

Ldb1:Isl1 activate *MafA*, *Arx*, and *Glp1r* transcription. To gain mechanistic insight into the linkage between Ldb1:Isl1 control in the developing pancreas, mRNA levels of many key islet-enriched transcriptional regulators were evaluated in E18.5 *Pax6-Cre;Ldb1*$^{F/F}$ pancreata (Fig. 4*A*). The expression of two known Isl1-controlled genes, *MafA* and *Arx* (22,23), were significantly reduced in the *Pax6-Cre;Ldb1*$^{F/F}$ pancreata (Fig. 4*A–C*). Isl1 regulates by binding to the *MafA* 5′-flanking region 3, *Arx* intronic Re1, and *Arx* 3′-flanking Re2 domains (15,22,23,45). Importantly, Ldb1 bound to these same transcriptional control sequences in ChIP experiments performed with βTC-3 (MafA$^+$) and αTC-6 (Arx$^+$) cells (Fig. 4*D*), providing compelling support for direct Ldb1:Isl1 complex activation. Endogenous Ldb1 and Isl1 were also found to interact in coimmunoprecipitation assays, whereas Ldb1 and Isl1 did not bind to the islet cell Pdx1, Pax6, NeuroD1, MafA, or Hnf1α transcriptional regulators (Fig. 4*E*). Moreover, dominant-negative acting Ldb1ΔN, which only produces the COOH-terminal LIM-interaction domain of Ldb1 (41), significantly reduced the activity of the Isl1-responsive *MafA* region 3-driven luciferase reporter in transfected βTC-3 cells (Fig. 4*F*). Collectively, these results demonstrate that specific interactions between Ldb1 and Isl1 are essential to expression of key endocrine genes like *MafA* and *Arx*.

To broaden our understanding of Isl1:Ldb1 regulation, we next analyzed in *Isl1*- and *Ldb1*-deficient pancreata the mRNA expression level of various Isl1 ChIP-sequencing (ChIP-Seq)–identified genes predicted to contribute to the deficiencies in islet cell formation and function in *Pdx1*^{late}*-Cre;Isl1*$^{F/F}$ mice (22). These candidates were selected from Isl1 ChIP-Seq data generated in βTC-3 cells (B.E., C.S.H., J. Liu, A. Du, E. Walp, R.S., C.L.M., unpublished observations). Intriguingly, only *glucagon-like peptide 1 receptor* (*Glp1r*) mRNA levels were significantly reduced in both the *Isl1* and *Ldb1* mutants (Fig. 5*A*). However, *Slc2A2* (encoding the

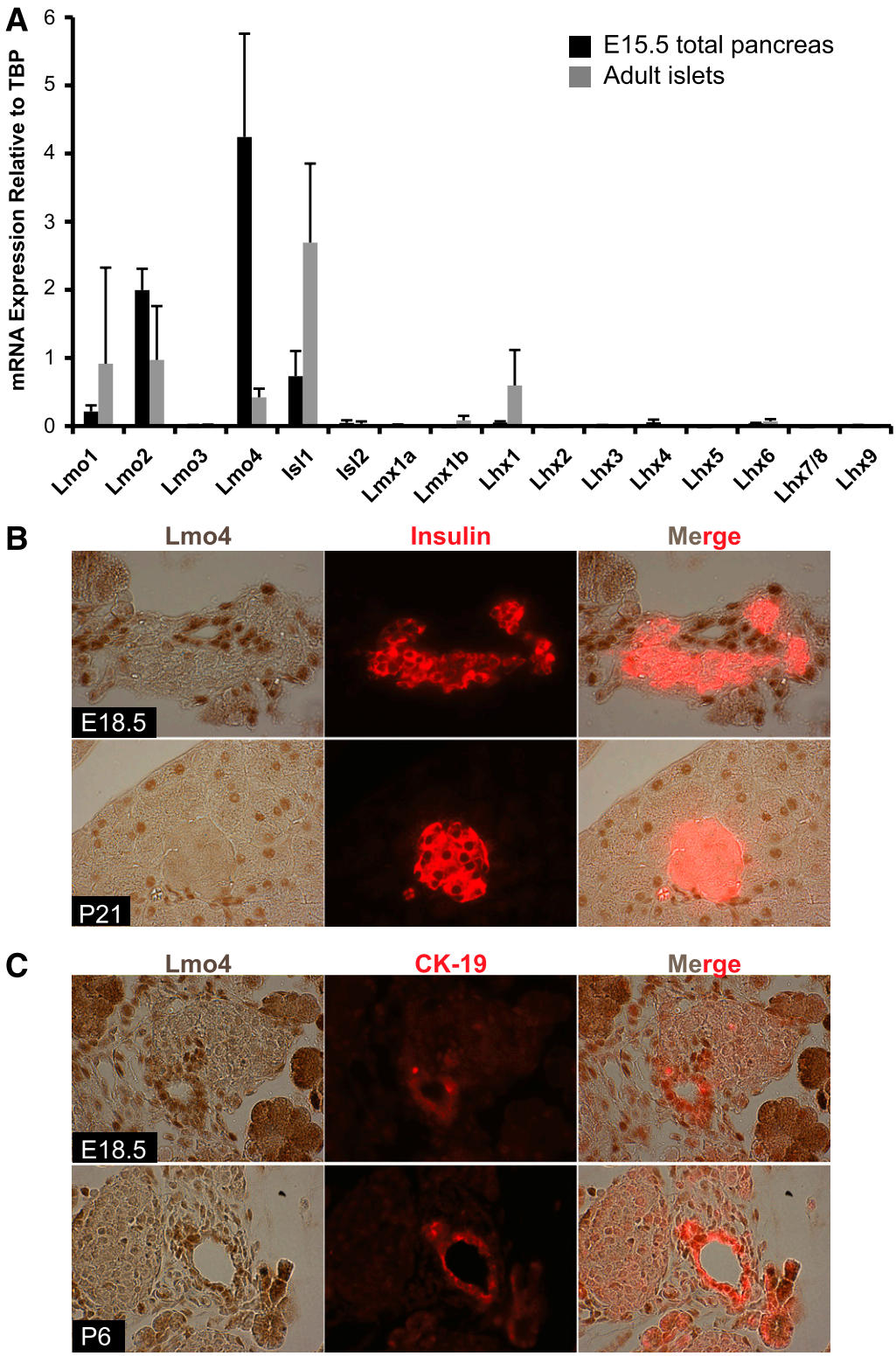

FIG. 2. Analysis of LIM-HD and Lmo levels in the developing and adult pancreas. *A*: LIM-HD and Lmo mRNA expression was measured by qPCR in the E15.5 pancreas (black bars) and 3-month-old islets (gray bars). Values are relative to TATA-binding protein (TBP), set at onefold. Error bars represent ± SEM (n = 5). *B*: Lmo4 (brown) and insulin (red) staining was performed on E18.5 and P21 pancreas tissue. Lmo4 was not found in hormone$^+$ cells. *C*: Lmo4 (brown) colocalized with the ductal CK-19 (red) marker in E18.5 and P6 pancreata. (A high-quality digital representation of this figure is available in the online issue.)

Glut2 glucose transporter) and *glucose-6-phosphatase 2* expression were exclusively decreased in the *Ldb1* mutant (Fig. 5*A*). Described below are immunohistochemical and ChIP data illustrating that *Glp1r* represents a new Ldb1:Isl1 activated gene (Fig. 5), whereas *Slc2A2/Glut2* appears to represent a class of genes dependent upon Ldb1 and not Isl1 (see section "Ldb1, and not Isl1, stimulates *Glut2* and *Hb9* expression during islet cell development").

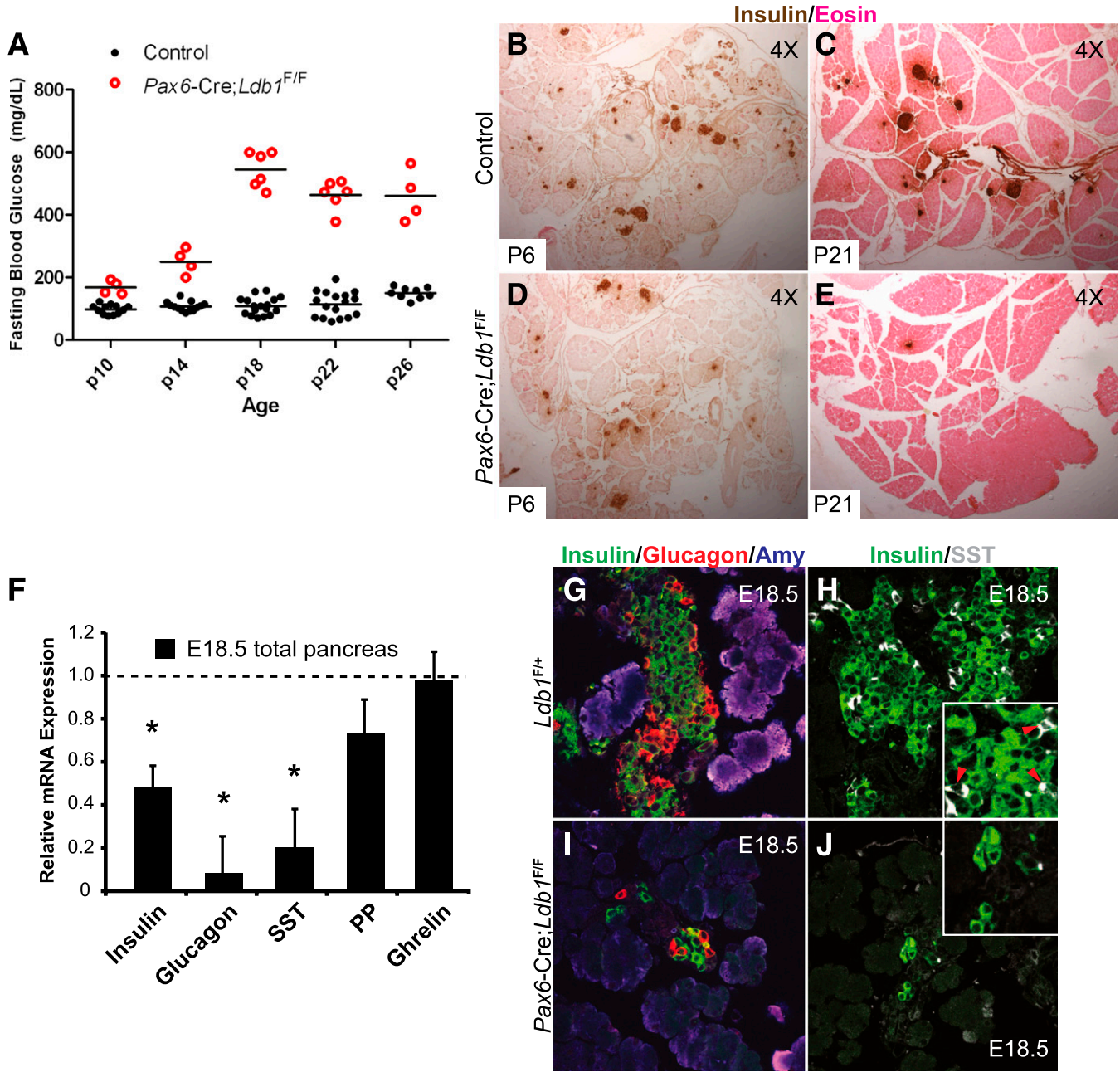

FIG. 3. Deletion of Ldb1 in endocrine hormone$^+$ cells causes reduced pancreatic hormone production, postnatal islet loss, and hyperglycemia in vivo. *A*: Six-hour fasting blood glucose levels in littermate control and *Pax6-Cre;Ldb1$^{F/F}$* pups. Horizontal bars indicate mean blood glucose values within each genotype, and values were significantly different between control and mutants at all ages ($P < 0.0001$). Insulin (brown) and eosin (pink) staining at P6 and P21 in the control (*B* and *C*) and *Pax6-Cre;Ldb1$^{F/F}$* (*D* and *E*) pancreas. *F*: Islet hormone *insulin*, *glucagon*, and *somatostatin* (SST) mRNA levels are significantly reduced in E18.5 Ldb1 mutant pancreata ($n = 4–6$). Data are presented relative to littermate controls, which are set at onefold and marked by the dashed line. Error bars represent ± SEM. The number of pancreatic insulin$^+$ (green), glucagon$^+$ (red), and somatostatin$^+$ (white) cells is greatly reduced between E18.5 control (*G* and *H*) and *Pax6-Cre;Ldb1$^{F/F}$* (*I* and *J*) tissue. The insets in *H* and *J* show a magnified view of the cell clusters. *$P < 0.05$. (A high-quality digital representation of this figure is available in the online issue.)

Glp1r is an important signaling hub for incretins like Glp1 and exendin in pancreatic β-cells and other extrapancreatic tissues (reviewed in Ref. 46). Glp1r and insulin protein staining was decreased in the β-cells of *Pax6-Cre;Ldb1$^{F/F}$* and *Pdx1^{Late}-Cre;Isl1$^{F/F}$* mice at P6 (Fig. 5*B* and *C*), paralleling their mRNA changes (Figs. 3*F* and 5*A*). There were four Isl1 ChIP-Seq binding peaks clustered between 16- and 10-kb pairs of the *Glp1r* start site (Fig. 5*D*). At least peak 1 (base pair −16,250 to −16,050) bound both Isl1 and Ldb1 (Fig. 5*E*), suggesting that these factors also act together to regulate *Glp1r* expression in developing β-cells.

Ldb1, and not Isl1, stimulates *Glut2* and *Hb9* expression during islet cell development. Glut2 is the major glucose transporter of islet β-cells (47). Strikingly, although the Re1 and Re2 control regions of *Slc2A2/Glut2* were Isl1-bound in βTC-3 cells (Fig. 6*C*), mRNA expression was only significantly reduced in the E18.5 pancreata of the *Ldb1* mutant (Fig. 5*A*). As expected, immunostaining revealed a discernible difference of this membrane protein from the control in the *Ldb1* mutant, but not the *Isl1* mutant (Fig. 6*A* and *B*). ChIP analysis was next performed over Isl1-bound Re1 and Re2 and the

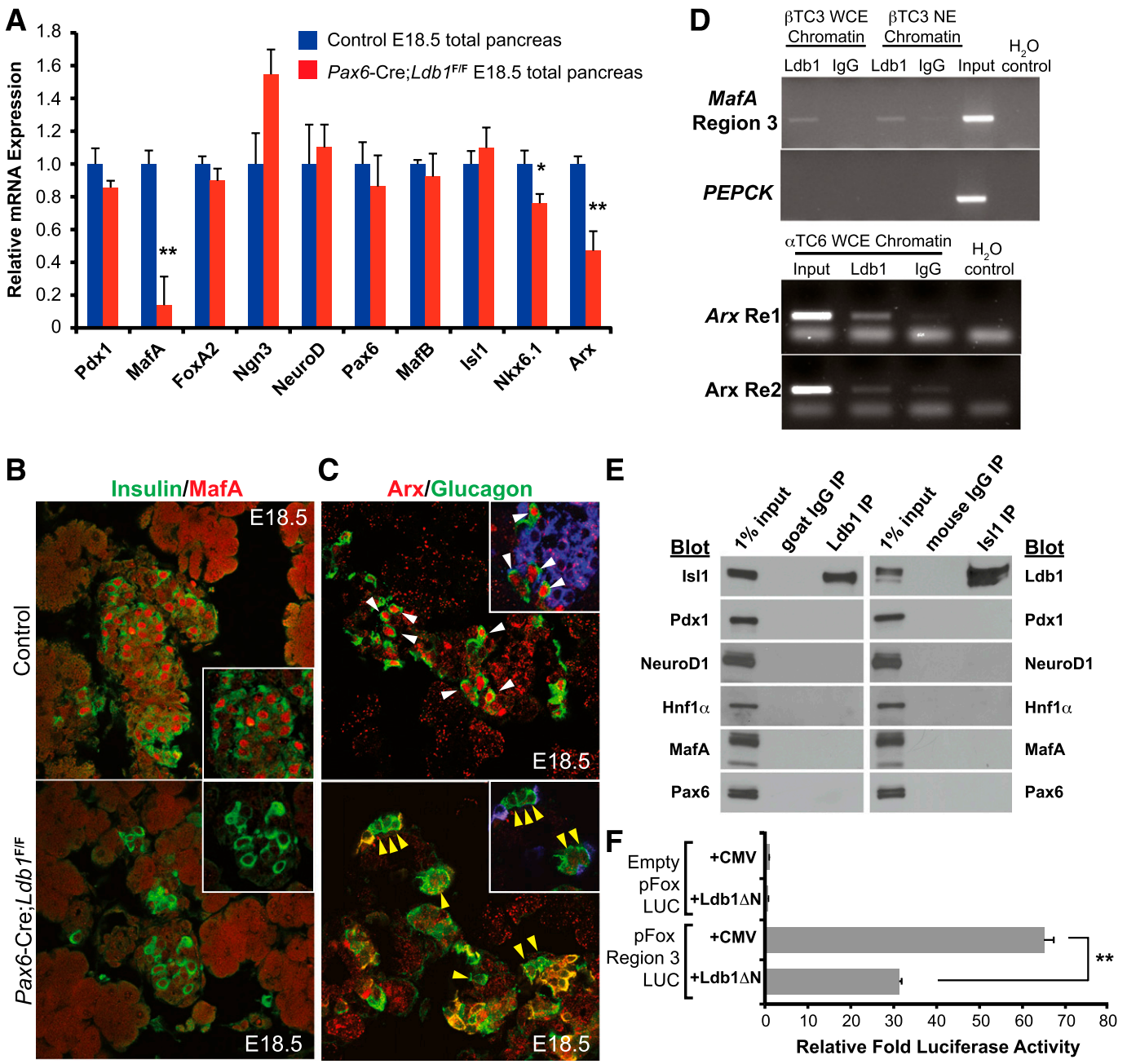

FIG. 4. Isl1-regulated *MafA* and *Arx* expression is greatly reduced in the E18.5 Ldb1 mutant pancreas. *A*: mRNA levels of islet-enriched transcription factors in E18.5 littermate control (blue bars) and *Pax6-Cre;Ldb1*$^{F/F}$ (red bars) pancreas (n = 4–6). Littermate control mRNA level was set at onefold ± SEM. Immunostaining levels of β-cell MafA (red) (*B*) and α-cell Arx (red) (*C*) were greatly reduced in E18.5 *Pax6*-Cre;*Ldb1*$^{F/F}$ pancreata. Arrowheads in *C* mark Arx^{+} glucagon^{+} (white, *top*) or Arx^{-} glucagon^{+} cells (yellow, *bottom*), with some magnified hormone^{+} cell clusters shown. *D*: ChIP analysis of Ldb1 binding to *MafA* Region 3 (*top*) as well as *Arx* Re1 and Re2 (*bottom*). The *PEPCK* promoter served as the negative background control. Dilute input as well as Ldb1- and IgG-enriched DNA were analyzed by PCR using βTC-3 and αTC-6 chromatin isolated from whole-cell extract (WCE) and/or nuclear extract (NE). H_2O control serves as a negative control for the PCR. *E*: Binding between endogenous Ldb1 and Isl1 were found in coimmunoprecipitation experiments using βTC-3 nuclear extracts, whereas Ldb1 and Isl1 did not bind to Pdx1, NeuroD1, Hnf1α, MafA, or Pax6. Diluted βTC-3 nuclear extract served as input positive control (1%), and immunoprecipitation (IP) results were compared with species-matched IgG treatments. *F*: Dominant-negative acting Ldb1ΔN significantly reduced *MafA* region 3-driven reporter expression in βTC-3 cells. Data are presented as mean fold reporter activity, with the empty pFox-Luc + CMV cotransfection set at onefold ± SEM; n = 3. *P < 0.05, **P < 0.01. Blot, immunoblot antibody probe. (A high-quality digital representation of this figure is available in the online issue.)

well-characterized proximal transcriptional control domain in βTC-3 cells (48,49). Interestingly, Ldb1 alone bound the proximal control domain, whereas both Isl1 and Ldb1 binding was detected within Re1 and Re2 (Fig. 6*D*). Collectively, these data suggest that Ldb1 and an unidentified LIM regulator(s) stimulate E18.5 *Glut2* transcription.

A complex composed of Ldb1, Isl1, and Lhx3 is essential to *Hb9* (also known as *Mnx1* and *Hlxb9*) expression in motor neurons (50), a transcription factor critical to both early pancreatic development (51) and motor neuron identity in the developing spinal cord (52). Because Isl1 binding was not found within or around the *Hb9* gene by ChIP-Seq analysis in βTC-3 cells (B.E., C.S.H., J. Liu, A. Du,

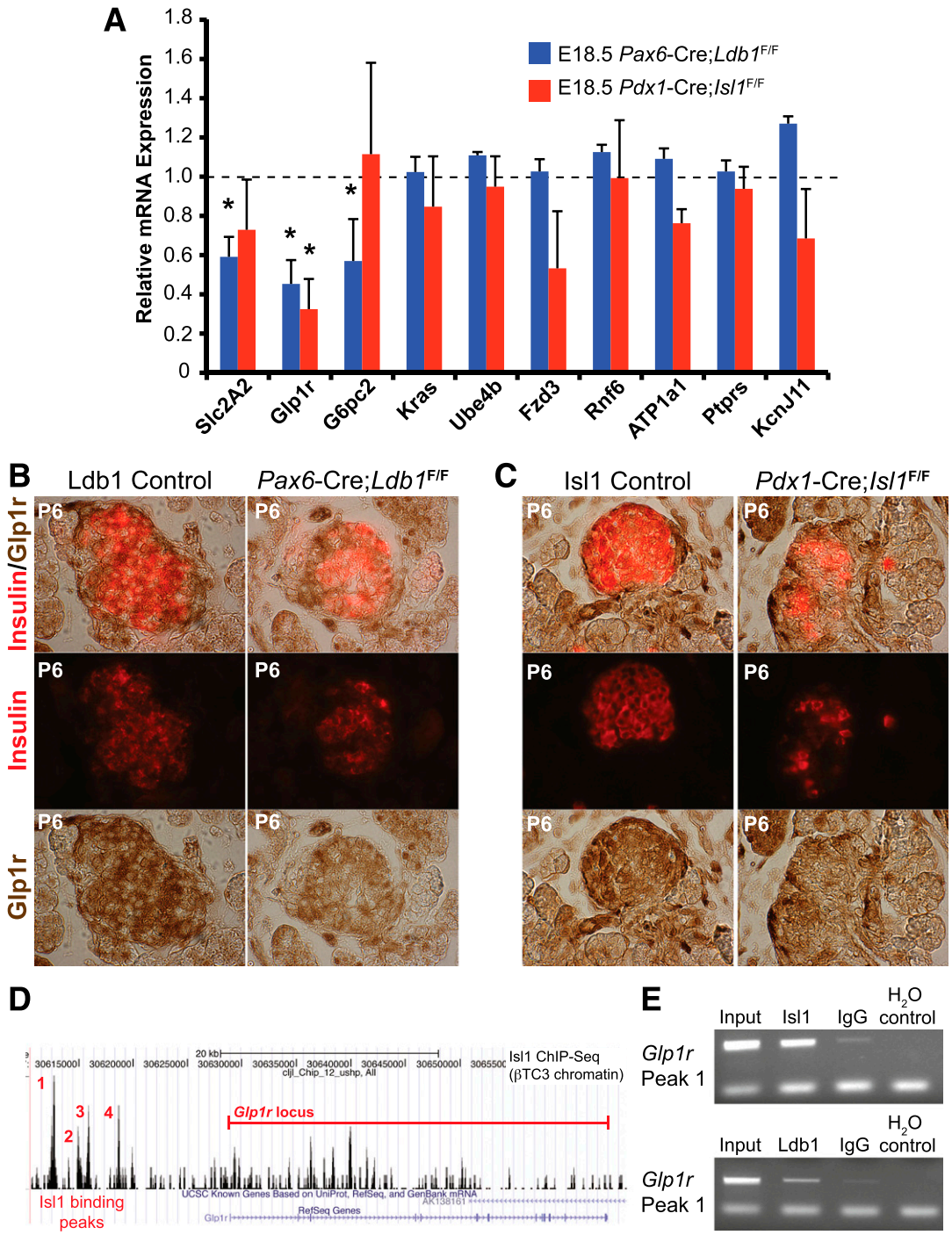

FIG. 5. ***Glp1r*** **is a novel Ldb1- and Isl1-activated target gene.** ***A*****: qPCR quantification of Isl1 ChIP-Seq candidates from Ldb1- (blue bars) and Isl1-deficient (red bars) E18.5 pancreata (*** n **= 4–6). Data are presented as fold of the littermate control, which was set at 1 (marked by the dashed line), ± SEM.** ***B*** **and** ***C*****: Immunostaining of Glp1r (brown) and insulin (red) at P6 illustrates reduced Glp1r protein levels in insulin$^+$ cells lacking Ldb1 or Isl1.** ***D*****: βTC-3 ChIP-Seq pictograph demonstrating the four distal 5′ peaks of Isl1 occupancy near** ***Glp1r*****. The red line denotes the** ***Glp1r*** **locus.** ***E*****: ChIP enrichment of peak 1** ***Glp1r*** **5′ DNA in Isl1 (*** ***top panel*** **) and Ldb1 βTC-3 immunoprecipitates (*** ***bottom panel*** **) as compared with IgG control-treated DNA. H_2O serves as a negative control for the PCR. *$P < 0.05$. (A high-quality digital representation of this figure is available in the online issue.)**

E. Walp, R.S., C.L.M., unpublished observations), it was not surprising that *Pdx1^{Late}-Cre;Isl1$^{F/F}$* pancreas mRNA and immunohistochemical staining levels at E18.5 were indistinguishable from wild-type (Fig. 6*E* and *F*). Nonetheless, Hb9 expression was significantly compromised in the *Ldb1* mutant (Fig. 6*E* and *G*). Because Ldb1 binding in β-cells could not be linked to *Hb9* control regions involved in either motor neuron (50) or islet β-cell transcription (53) by ChIP, it remains unclear if Ldb1 directly or indirectly stimulates expression.

DISCUSSION

Regulation of gene transcription involves the recruitment of coregulators by DNA-bound transcriptional activators and repressors. These protein–protein interactions ultimately

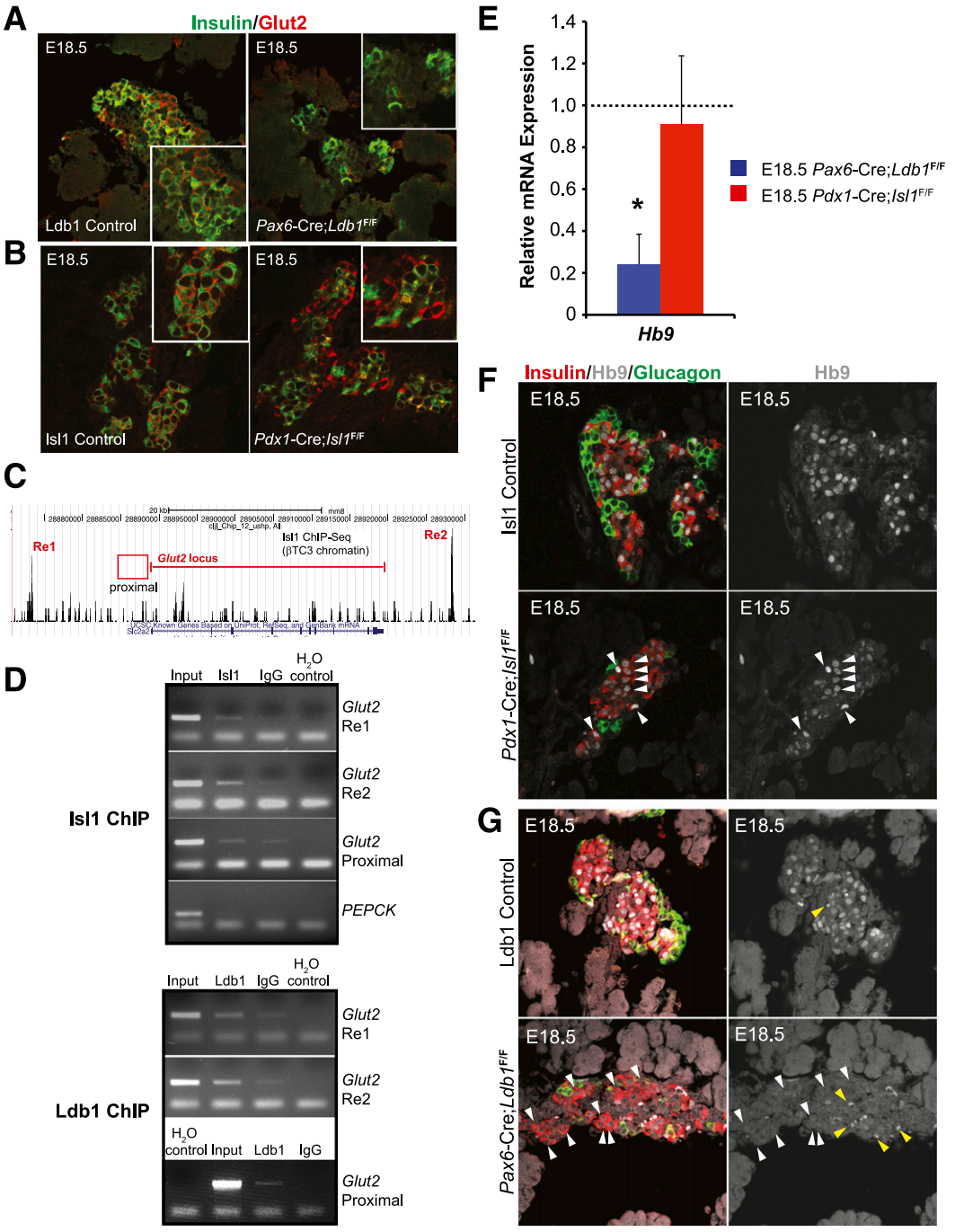

FIG. 6. E18.5 *Glut2* and *Hb9* mRNA and protein expression is only compromised in Ldb1 mutant mice. Immunofluorescence analysis of Glut2 (red) and insulin (green) in the E18.5 control, mutant Ldb1 (*A*), and mutant Isl1 (*B*) pancreas. Insets show magnified insulin$^+$ cell clusters. *C*: ChIP-Seq pictograph demonstrating Isl1 occupancy at distal *Glut2* Re1 (5′) and Re2 (3′) domains in βTC-3 cells. The red line denotes the Glut2-coding region, whereas ChIP-tested proximal 5′ promoter region is represented by the red box. *D*: βTC-3 ChIP analysis of Isl1 (*top panel*) and Ldb1 (*bottom panel*) occupancy of *Glut2* Re1, Re2, and the proximal domain compared with the *PEPCK* control (from *top* to *bottom*, respectively). H_2O serves as a negative PCR control. Results recapitulate observed Isl1 ChIP-Seq occupation of *Glut2* Re1 and Re2, whereas Ldb1 also binds to the proximal domain. *E*: qPCR analysis of E18.5 *Hb9* mRNA levels in pancreata from Ldb1- (blue bar) and Isl1-deficient (red bar) pancreata. Littermate control mRNA level was set at onefold (dashed line) ± SEM. *F*: E18.5 immunostaining analysis demonstrates that Hb9 protein (white) is maintained in the insulin$^+$ (red) nuclei of *Pdx1-Cre*;*Isl1$^{F/F}$* pancreata as compared with littermate controls, as denoted by the white arrowheads. *G*: However, Hb9 is lost from most remaining insulin$^+$ cells in the *Pax6-Cre*;*Ldb1$^{F/F}$* pancreata seen by comparing white arrowhead–labeled Hb9$^+$ cells of control and mutant in *F* and *G*. The nuclear Hb9 signals are shown in the *right panels*. Yellow arrowheads in *G* illustrate autofluorescence from erythrocytes. **P* < 0.05. (A high-quality digital representation of this figure is available in the online issue.)

influence the activity of the RNA polymerase II transcriptional machinery. We conclude from the analysis of pancreatic endocrine cell knockout mice in this study that the LIM-HD and Lmo coregulator Ldb1 is specifically required in α-, β-, and δ-cell production from islet progenitors during development. The inability of these cell types to properly form leads to overt hyperglycemia soon after birth. These findings were very similar to those observed upon removal of Isl1 from *Pdx1^{Late}-Cre*;*Isl1$^{F/F}$* mice pancreata, the predominant LIM-HD transcription factor in the developing

and adult pancreas. However, Ldb1 also likely mediates activation by other LIM-HD and Lmo transcription factors, as suggested both by its much broader expression than Isl1 in very early pancreatic Pdx1$^+$ dorsal and ventral lobe cell populations and unique impact in *Hb9* and *Glut2* transcription in later hormone$^+$ cell formation. Clearly, the mechanisms by which Ldb1 controls pancreatic cell development and function warrant further investigation.

The Ldb1 and Ldb2 coregulators lack DNA-binding, transactivation, and enzymatic capacity. In contrast, the few other characterized coregulators associated with islet-enriched transcription factors possess enzymatic capabilities. For example, Pdx1 recruits coregulators capable of catalyzing modifications influencing protein stability [PDX-1 COOH terminus–interacting factor 1 (29)] and epigenetic control [CBP/p300 (32–34) and Set7/9 (30,48)]. Ldb1 and Ldb2 act by binding to LIM domain proteins through their COOH-terminal LIM interaction domain to regulate the stoichiometry, positioning, and abundance of Ldb-LIM complexes on target gene promoters (50,54,55). Notably, Isl1-dependent *MafA* region 3-driven activation was inhibited by a dominant-negative Ldb1 mutant spanning this COOH-terminal interaction surface (Fig. 4*F*). Although Ldb1 is important in developing heart (44), spinal cord (50), pituitary gland (26), and limb (36,56), the studies in this paper were the first to examine Ldb1 and Ldb2 expression and function in the developing pancreas.

Ldb1 was widely produced in the developing pancreas by E10.5, including throughout the Pdx1$^+$ pancreatic epithelium and surrounding mesenchyme (Fig. 1). Later in development, Ldb1 became enriched in islet and ductal cells, with much lower expression in the exocrine pancreas. *Ldb2* mRNA was also expressed in the pancreas and many surrounding tissues at E15.5, although the cellular protein distribution is unclear due to lack of immunostaining reagents. Notably, our analysis of *Ldb1* (Fig. 3) and *Ldb2* (Supplementary Fig. 2) mutant mice demonstrated that only Ldb1 was impactful to endocrine hormone$^+$ cell production, specifically insulin-, glucagon-, and somatostatin-expressing cells. Because *Ldb2*$^{-/-}$ animals appear overtly normal [Mouse Genome Informatics, Lexicon Genetics (36)], in stark contrast to *Ldb1*$^{-/-}$ (44), it was not surprising to find that Ldb2 was of lesser (if any) relevance. The low abundance of *Ldb2* mRNA compared with *Ldb1* further suggests that Ldb2 has a limited (if any) functional role in adult islet cells (Fig. 1).

Pax6-Cre specifically eliminated Ldb1 expression from the majority of hormone$^+$ cells in *Ldb1*$^{F/F}$ mice by E15.5 and compromised all but islet PP and ε (i.e., ghrelin$^+$) cell formation, glycemic control, and viability (Fig. 3, data not shown). The widespread impact was analogous to removal of Isl1 from the developing pancreas with *Pdx1*^{Late}*-Cre* (22). Thus, both Ldb1 and Isl1 activated critical effectors of islet α-cell [*Arx* (23)] and β-cell (*MafA* and *Glp1r*) formation and/or function (Fig. 4) (22). For example, Ldb1:Isl1 regulation of *Glp1r* was established by ChIP-Seq and mRNA expression analysis in E18.5 *Pdx1*^{Late}*-Cre*;*Isl1*$^{F/F}$ and *Pax6-Cre*;*Ldb1*$^{F/F}$ pancreata (Fig. 5), with loss of Glp1r synthesis likely also contributing to compromised β-cell mass and function in the *Isl1* and *Ldb1* mutants (57).

Whereas Isl1 was the major LIM-HD factor expressed in the pancreas, *Lmo1*, *Lmo2*, and *Lmo4* mRNA were produced in E15.5 pancreas and adult islets (Fig. 2*A*). *Lmo1* and *Lmo2* mRNA levels are also islet-enriched in humans (42). Binding of Lmo2 factors to Gata1/2, Tal1/Scl (basic helix-loop-helix), and Ldb1 is essential to α- and *β-globin* transcription during erythropoiesis (54,58). Existing antibody reagents limited our analysis only to Lmo4 in the pancreas, which was found enriched in ductal and acinar cells (Fig. 2*B* and *C*) (42). This distribution pattern is quite distinct from endocrine cell–enriched Isl1, suggesting that interactions among Ldb1, Lmo4, and exocrine-enriched Gata4 (59) could be critical to ductal and acinar cell formation. Moreover, regulatory complexes containing Ldb1, endocrine cell–enriched Gata6 (59,60), and Lmo1/2 could potentially contribute to *Hb9*, *Nkx6.1*, and *Glut2* transcription during development, which represent genes unaffected in the E18.5 *Isl1*-deficient pancreata (Figs. 4 and 6). However, the impactful nature of *Isl1* loss in the pancreas of the *Pdx1*^{Late}*-Cre*;*Isl1*$^{F/F}$ mice on α-, β-, and δ-cell formation implies that such complexes are of reduced regulatory importance. In contrast, the relative abundance of Ldb1 to Isl1 in the Pdx1$^+$ cells of the E10.5 dorsal and ventral pancreatic buds strongly indicates that distinct LIM-HD and Lmo proteins will be essential to Ldb1-mediated production of these pancreatic progenitor cells (Fig. 1).

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C.S.H. researched data and wrote the manuscript. S.D., T.C., B.E., C.W., and M.F. researched data. H.W. reviewed and edited manuscript. R.S. and C.L.M. supervised research and wrote the manuscript. R.S. and C.L.M. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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