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The Ldb1 transcriptional co-regulator is required for establishment and maintenance of the pancreatic endocrine lineage

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Abstract

Pancreatic islet cell development is regulated by transcription factors (TFs) that mediate embryonic progenitor differentiation toward mature endocrine cells. Prior studies from our lab and others showed that the islet-enriched TF, Islet-1 (Isl1), interacts with the broadly-expressed transcriptional co-regulator, Ldb1, to regulate islet cell maturation and postnatal function (by embryonic day (E)18.5). However, Ldb1 is expressed in the developing pancreas prior to Isl1 expression, notably in multipotent progenitor cells (MPCs) marked by Pdx1, and endocrine progenitors (EPs) expressing Neurogenin-3 (Ngn3). MPCs give rise to the endocrine and exocrine pancreas, while $Ngn3^{+}$ EPs specify pancreatic islet endocrine cells. We hypothesized that Ldb1 is required for progenitor identity in MPC and EP populations during development to impact islet appearance and function. To test this, we generated a whole-pancreas Ldb1 knockout, termed $Ldb1^{\Delta Panc}$, and observed severe developmental and postnatal pancreas defects including disorganized progenitor pools, a significant reduction of Ngn3-expressing EPs, $Pdx1^{HI}$ β-cells, and early $hormone^{+}$ cells. $Ldb1^{\Delta Panc}$ neonates presented with severe hyperglycemia, hypoinsulinemia, and drastically reduced hormone expression in islets, yet no change in total pancreas mass. This supports endocrine-specific actions of Ldb1. Considering this, we also developed an endocrine-enriched model of Ldb1 loss, termed $Ldb1^{\Delta Endo}$. We observed similar dysglycemia in this model, as well as a loss of islet identity markers. Through *in vitro* and *in vivo* chromatin immunoprecipitation experiments, we found that Ldb1 occupies key *Pdx1* and *Ngn3* promoter domains. Our findings provide insight into novel regulation of endocrine cell differentiation that may be vital toward improving cell-based diabetes therapies.

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Keywords

pancreatic development; islets; diabetes; endocrine; cell fate; transcription; transcription factor; co-regulator

Introduction

With an estimated 34 million cases in the US (13% of the adult population), diabetes is a growing epidemic that poses a significant threat to not only national, but global public health (1). Diabetes is broadly defined by the loss of functional insulin producing β-cells via autoimmune destruction in Type 1, and by insulin resistance associated with β-cell dysfunction and dedifferentiation in Type 2. As disease complexity continues to unfold, there is a growing appreciation for β-cell dysfunction mechanisms in both pathologies (2). Concomitantly, diabetes can be defined as a bi-hormonal disease due to hyper-secreting glucagon-producing α-cells and thus lack of postprandial glucagon suppression in diabetic patients (3). Both cell types reside within the pancreatic islets of Langerhans, clusters of cells embedded in the exocrine pancreas that cohesively function in endocrine and paracrine fashions to sense and tightly regulate glucose homeostasis (4–6). While β-cells are the most abundant cell in most mammalian islets, islets are comprised of five different hormone-secreting cell types (α, β, δ, ε, and pancreatic polypeptide (PP)) that are intimately linked in structure and function (7, 8). Insulin produced by β-cells acts to decrease blood glucose levels by increasing glucose uptake in peripheral tissues, while glucagon produced by α-cells increases blood glucose levels to prevent hypoglycemia (9). The less abundant δ, ε, and PP cells produce the hormones somatostatin, ghrelin, and PP respectively. In addition to progress toward differentiating replacement β-cell mass *de novo* (10–12), there are new efforts to also generate other islet cell types in order to more comprehensively mimic the islet environment. Future diabetes therapies require a deep knowledge, not only of the mature function of islet cell types, but of the gene regulatory mechanisms that characterize their developmental origins.

Key to the differentiation of islet progenitors are the transcription factors (TFs) that dictate gene expression cascades throughout the cell fate process. In the pancreas, this process is broadly divided into stages defined as the primary and secondary transitions. During the primary transition (i.e., embryonic day (E) 9.5–12.5), the homeodomain (HD) TF Pdx1 is the first to demarcate the multipotent progenitor cells (MPCs) of the pancreatic buds (13). MPCs are highly proliferative stem-like progenitors that will populate the pancreatic buds before cell fate specification occurs. Strikingly, Pdx1 loss results in pancreatic agenesis in mice and humans (13, 14). Subsequently, the secondary transition (i.e., E12.5-E16.5) is characterized by tightly regulated segregation of MPCs into their separate identity pools. A subset of MPCs fated to become endocrine progenitors (EPs) will transiently express the helix-loop-helix TF Neurogenin-3 (Ngn3), starting around E12.5 and peaking at E14–15.5. $Ngn3^{+}$ EPs will exit the ductal epithelium, where they aggregate to form islet clusters, giving rise to all islet endocrine cell types (15, 16). Consequently, mice with a targeted *Ngn3* disruption still form a pancreas, but specifically fail to produce islets. The subset of EPs fated to become β-cells will express high levels of Pdx1 ($Pdx1^{HI}$), prior to progressing

to insulin positivity (17, 18). Control of *Pdx1* expression has been extensively studied, with TFs acting in four conserved 5′ *cis*-regulatory regions (termed Areas I-IV) upstream of the transcriptional start site (TSS) (19, 20). Areas I-III influence expression during embryonic development and can rescue pancreatic defects caused by Pdx1 deficiency (21). More specifically, the mammalian Area II is required for endocrine fate selection and β-cell maturation and Area IV deletion affects later β-cell function and growth after weaning (22, 23). These regulatory regions act as transcriptional hubs for pancreatic development and function, occupied by a multitude of TFs including MafA, FoxA1, Pax6, and Pdx1. Although, much less characterized, *Ngn3* regulation appears to be controlled by at least six conserved regions 5kB upstream of the TSS that are occupied and regulated by Pdx1 (24, 25).

Despite extensive characterization of TFs in the pancreas, only a small handful of transcriptional co-regulators have been identified and explored in the islet, and even fewer during cell fate specification (26, 27). The Swi/Snf chromatin remodeling complex directly associates with Pdx1 during organogenesis and onward, with disruption of this interaction causing glucose intolerance and impaired insulin secretion (28). The Set7/9 histone methyltransferase is enriched in islets and interacts with Pdx1, also allowing for maintenance of Pdx1 activity in β-cells (29, 30). Interaction with P300/CBP is also required for Pdx1 activation, while MLL3/4 association with MafA and MafB TFs is necessary for transactivation both during development and postnatally (31, 32). Transcriptional co-regulators have been largely unexplored in cell-based therapeutic strategies and are likely to be as vital to controlling cell fate as the TFs themselves.

Our lab and others have shown that the co-regulator Ldb1 (LIM domain binding protein 1) is required for expression of critical islet cell genes in the developing and adult pancreas (33, 34). Ldb1 interacts with LIM-domain (named for Lin11-Isl1-Mec3) TFs, including LIM-HD and LMO (LIM-only) factors and has been implicated in broad developmental contexts, including neurogenesis and hematopoiesis (35, 36). *Ldb1*$^{-/-}$ embryos do not develop beyond ~E9.5 due to severe head truncation, heart anlage, and axis duplication defects (37). Rather than direct DNA-binding or chromatin modification, Ldb1 acts as a dimerized scaffold for larger LIM TF complexes (38). In the pancreas, Ldb1 and Isl1 interact to co-regulate key islet genes including, *insulin*, *MafA*, *Glp1r*, and *Arx* (33). However, Ldb1 is highly expressed in the earliest populations of the developing pancreas mentioned above, MPC and Ngn3^{+} EPs, which precede Isl1 expression (33, 39). We hypothesized that Ldb1 is required for maintenance of the MPC and EP populations during development to impact islet appearance and function.

Here, we show that Ldb1 has vital roles in the endocrine progenitors of the pancreas. Mice lacking Ldb1 beginning in MPCs exhibit overt reductions in islet hormone^{+} cells and do not survive beyond the neonatal period (~P7). Ldb1 is required for the appearance and differentiation of the Ngn3^{+} islet progenitor pool, as well as subsequent nascent β-cells, marked by Pdx1^{HI} expression. Furthermore, overtly unchanged pancreas size and analysis of a *Ngn3*-driven EP-specific knockout reveal that Ldb1 acts in an endocrine-specific manner in the developing pancreas. We find that these effects are likely mediated through direct regulation of both *Pdx1* and *Ngn3*. Our results highlight a unique regulatory role for Ldb1

as a vital player in the differentiation of early pancreatic progenitors towards endocrine cell fate.

Methods

Animals

Previously described *Ldb1*^{Flox} (40) and *Ldb1* null allele (37) mice were maintained on a B6 background. *Pdx1-* (41) and *Ngn3-Cre* (42) lines were also maintained on a B6 background to generate pancreas and islet-wide knockouts, respectively. *Ldb1*$^{F/F}$*; Pdx1-Cre* or *Ldb1*$^{+/-}$*; Pdx1-Cre* males were crossed with global heterozygous *Ldb1*$^{F/-}$ or *Ldb1*$^{F/F}$ females (no overt developmental phenotype (43)) to generate *Ldb1*$^{F/-}$*; Pdx1-Cre* mutants (termed *Ldb1*$^{\Delta Panc}$), heterozygous (Ldb1$^{F/-}$ and *Ldb1*$^{F/+}$*; Pdx1-Cre*), and control animals (*Ldb1*$^{F/F}$). Due to *Pdx1-Cre* mosaicism (44), the *Ldb1* null allele was used to enhance Ldb1 loss, an approach used by other investigators (45). The *Ldb1*$^{F/F}$*; Ngn3-Cre* mice do not all survive to adulthood, thus a mating scheme using *Ldb1*$^{+/-}$*; Ngn3-Cre* males and *Ldb1*$^{F/F}$ females was used to generate *Ldb1*$^{F/-}$*; Ngn3-Cre* (termed *Ldb1*$^{\Delta Endo}$), mutants and littermate heterozygotes (*Ldb1*$^{F/-}$, *Ldb1*$^{F/+}$; *Ngn3-Cre*, *Ldb1*$^{+/-}$*; Ngn3-Cre*) and controls (*Ldb1*$^{F/+}$). Embryos were collected as early as E11.5, where the morning of vaginal plug detection was considered E0.5. CD1 mice (Charles River Labs (46)) were used for embryonic chromatin preparation due to large litter sizes. All studies were approved by and performed in accordance with the guidelines of the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Postnatal Glucose Physiology and Pancreatic Mass Index

Trunk blood was collected from postnatal day 1 (P1) animals and blood glucose was measured using an automatic glucometer (Bayer Contour Next). Plasma was also collected and flash frozen for insulin measurements by ELISA (Crystal Chem # 90080) according to manufacturer's instructions. Pancreata were collected, weighed for pancreatic mass index calculation (pancreas weight/total body weight x 100), and fixed for histology.

Immunohistochemical Analysis and Quantification

Embryonic pancreata were isolated, fixed in 4% formaldehyde/PBS for 2–4 hrs (postnatal pancreata were fixed overnight), and embedded in paraffin. Sections were cut to 6 μm using a Leica RM2235 microtome and blocked using 5% normal donkey serum (Jackson ImmunoLabs) in 1% bovine serum albumin/PBS for 1 hr at room temperature. Slides were then incubated with primary antibodies at 4°C overnight (see Supplemental Table 1 for sources and dilutions). Secondary antibodies were used to detect immunofluorescence for 2 hrs at room temperature, then slides were mounted using DAPI Fluoromount G mounting medium (Southern Biotech # 0100–20). Imaging was performed using an Olympus IX81 fluorescence or Zeiss LSM710 confocal microscopes and processed by CellSens Dimension version 1.12 (Olympus) or Zen Software (Zeiss). For quantification at E13.5, the whole pancreas was cut and every other section was counted for a total of up to 290 Ngn3^{+} cells per pancreas (modified from (23)). At E15.5, a ratio of Pdx1^{HI}/Nkx6.1^{+} trunk cells was calculated by counting Pdx1 and Nkx6.1 (denoting "trunk") cells (47) in at least three sections per embryonic pancreas. Ngn3^{+} cells were counted across genotypes in three

identically-sized selected areas that encompassed the pancreatic epithelium, denoted in figure as “epithelial area.” All counting was performed in a blinded fashion.

RNA Isolation, cDNA Synthesis, and qPCR Analysis

E15.5 embryonic pancreata were microdissected and stored in RNA Later (AM7021; Invitrogen). RNA was isolated using an RNeasy Micro Kit (Qiagen, #74004) and cDNA was synthesized using the iScript cDNA kit (Bio-Rad, #170–8840). qPCR was performed in duplicate using iTaq SYBR Green (Bio-Rad, #172–8840) and Bio-Rad CFX96 C1000 Touch Real-Time PCR Detection System. Data was analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to *gapdh* as the housekeeper (primer sequences available in Supplemental Table 2).

Chromatin Immunoprecipitation (ChIP)

Cell line chromatin was prepared as previously described (33, 48, 49). Embryonic chromatin was prepared using E18.5 wildtype CD1 embryos. 8–12 whole pancreata per prep were microdissected, flash frozen, sheared (22g needle), and solution crosslinked using 2% formaldehyde in PBS for 10 min at room temperature. Chromatin fragments were generated by sonication using the Diagenode Bioruper Pico and precleared using Protein-G Dynabeads (10004D; Invitrogen). ChIP was then performed using EZ-Magna ChIP A/G Kit (Millipore, #17–10086). Briefly, chromatin aliquots were incubated with A/G beads and anti-Ldb1 (ab96799; Abcam or sc11198x; Santa Cruz) or rabbit IgG (P120–101; Bethyl Laboratories) antibodies at 4°C overnight with rotation. Bound complexes were washed and crosslinks were reversed, DNA was purified. qPCR was performed on immunoprecipitated DNA using SYBR Green PCR master mix (Bio-Rad) and primers for *Pdx1* (19) and *Ngn3* (24) regulatory sequences (Supplemental Table 2). For cell line ChIP, enrichment of target control sequences in ChIP DNAs was normalized to inactive *albumin* control sequences (50) and calculated relative to rabbit IgG enrichment set as one-fold (ΔΔCt). Enrichment of target regions for *in vivo* ChIP was calculated as percent input (as described (51)) and plotted relative to rabbit IgG enrichment (set to one-fold).

Cell Transfection and Reporter Gene Assays

siRNA transfection and gene reporter assays were performed as described (48). Briefly, Min6 cells were seeded in 6-well tissue culture dishes and transfected with 50nM On-Target Plus Smart Pool Ldb1 targeted (siLdb1, L-043882-00; GE Healthcare/Dharmacon) or scrambled (siSCR, D-001810-10; GE Healthcare/Dharmacon) using RNAiMax (13778030; Life Technologies). Immunoblotting was performed 48 hours after transfection. Cells were co-transfected with 1 ug of *Pdx1* Area II luciferase plasmid (kind gift from R. Stein, Vanderbilt) or luciferase control empty vector and siLdb1/siSCR duplexes. After 48 hours, whole cell lysates were prepared to assess luciferase activity using the Dual Luciferase assay kit (Promega, E1910). Each experiment was performed in triplicate on at least three independent occasions.

Statistical Analysis

All data are represented as mean ± SE. Statistical significance was determined using a Student's *t*-test or a two way ANOVA, followed by post hoc analysis using GraphPad Prism statistical software (version 9.3). A *P* value of less than 0.05 was considered significant.

Results

Ldb1$^{\Delta Panc}$ neonates exhibit severe glucose dysregulation

To investigate the role of Ldb1 in pancreatic progenitor differentiation, we used a *Pdx1-Cre* driven approach (Fig. 1A) where *Ldb1* was recombined in the pancreatic anlage by E11.5, as indicated by Pdx1 and Ldb1 co-immunofluorescence (Fig. 1B-E). Litters were born at expected Mendelian ratios and at P1, *Ldb1*$^{\Delta Panc}$ pups who had fed (indicated by a milk spot) were significantly hyperglycemic (Fig. 1F, left), and unfed pups lacking a milk spot were hypoglycemic (Fig. 1F, right), suggesting an inability to regulate blood glucose levels in both feeding states. The hyperglycemia prompted us to measure plasma insulin levels, which were reduced in mutant neonates (Fig. 1G). Interestingly, we found no change in overall pancreatic mass in *Ldb1*$^{\Delta Panc}$ mice (Fig. 1H-J) and amylase expression and P1 acinar morphology appeared unchanged (data not shown). A lack of observable changes in the exocrine compartment highlight that, despite whole-pancreas knockout, Ldb1 roles in the acinar lineage may be minor.

Ldb1 is required for hormone expression and β-cell identity

We performed immunofluorescence at P5 and observed a striking lack of islet structures, with only a few hormone^{+} cells remaining in the *Ldb1*$^{\Delta Panc}$ mutant mice and no observable changes to amylase staining (Fig. 2A-B). ChromograninA (chgA) staining revealed that despite severe hormone loss, many chgA^{+} cells remain in mutant tissues (Supplemental Fig. 1A-B). The few remaining islet cells appeared to lack markers of β-cell functional identity including TFs Pdx1 and maturity marker MafA, as well as glucose transporter GLUT2 (Fig. 2C-F). Some of the hormone^{-}/MafA^{-} cells still maintain Nkx6.1 expression (white arrows, Fig. 2F). *Ldb1*$^{\Delta Panc}$ mice did not survive past P7 (data not shown). The dramatic hormone cell loss and unchanged pancreatic mass suggests that, despite a knockout in MPCs that populate the entire pancreas, roles for pancreatic Ldb1 may be endocrine-specific.

Ldb1 is required for early pancreatic progenitor identity

We next sought to explore early stages of endocrine cell differentiation to determine the timing and mechanism for roles of Ldb1 in pancreatic progenitors, starting with tip/trunk specification (the first step of the secondary transition) (52). In control tissues, Ldb1 is broadly expressed and we found little-to-no overlap of tip and trunk markers, Ngn3 (trunk) and carboxypeptidase-1 (Cpa1, tip), as expected for these separate identity domains (Fig. 3A,C (52)). In the *Ldb1*$^{\Delta Panc}$ mutants, we found a strong trend toward increased occurrences of Ldb1^{-} cells that co-express Ngn3 and Cpa1, suggesting a defect in early fate choice of the MPC population upon loss of Ldb1 (Fig. 3B,D-E). Additionally, we observed a trend towards decreased appearance of Ngn3^{+} endocrine progenitors at E13.5 (Fig. 3F). These

data support a potential role for Ldb1 in specification early pancreatic progenitors towards proper endocrine cell fate.

Ldb1 is necessary for appearance of key islet progenitor populations

We next examined *Ldb1*$^{\Delta Panc}$ and control mice at E15.5, which is a peak of Ngn3 expression, but also when some endocrine progenitors have progressed to hormone expression. At this stage, we found that Ngn3^{+} cell numbers were significantly reduced in the *Ldb1*$^{\Delta Panc}$ embryos (Fig. 4A-C, yellow arrows), highlighting a reduction in the key endocrine progenitor population. We also examined Pdx1^{HI} cells by immunofluorescence, a marker of presumptive β-cells (17, 18). Pdx1^{HI} cells present as intensely bright nuclei compared to lower Pdx1 nuclear signals in (for example) the exocrine pancreas (Fig. 4A-B, white arrows). These future β-cells were significantly reduced upon Ldb1 loss (Fig. 4D). Hormone^{+} cells were also assessed at this time point by immunofluorescence and insulin, glucagon, and somatostatin cells were overtly reduced in mutant tissues (Fig. 4E-F). No co-expression of hormones was observed in any remaining hormone^{+} cells. Hormone-encoding mRNAs from whole E15.5 pancreata were also reduced, highlighting a loss of *insulin*, *glucagon*, and *somatostatin* in the developing pancreas (Fig. 4G). *Ghrelin* mRNA was unchanged, and histology supports that ghrelin^{+} cells are unaffected by Ldb1 loss (Supplemental Fig. 2).

Islet-specific loss of Ldb1 in an *Ldb1*$^{\Delta Endo}$ model recapitulates defects observed in *Ldb1*$^{\Delta Panc}$ mice

Considering the apparent endocrine-specific defects observed in the *Ldb1*$^{\Delta Panc}$ knockout, we next developed an islet-specific model as a targeted tool to assess Ldb1 roles in EPs. We used a *Ngn3-Cre* driven system to create the *Ldb1*$^{Flox/-}$*; Ngn3-Cre* (*Ldb1*$^{\Delta Endo}$) model. We show that Ldb1 is lost specifically in the cells that populate the islet, but not pancreatic acinar or ductal cells (Fig. 5A-B). As expected, pancreatic mass and neonatal body weight were unchanged in these mice (Fig. 5C, data not shown). We found that P1 *Ldb1*$^{\Delta Endo}$ mice, like *Ldb1*$^{\Delta Panc}$, were hyperglycemic under fed conditions and that plasma insulin levels were significantly reduced, compared to littermate controls (Fig. 5D-E).

While some hormone^{+} cells were detectable by immunofluorescence, we observed severe reductions in the presence of islet hormones (Fig. 6A-B). Scattered insulin^{+} cells remain, but we observe almost complete loss of somatostatin^{+} and glucagon^{+} cells (Fig. 6B). We assessed the remaining cells in *Ldb1*$^{\Delta Endo}$ for TFs Pdx1 and Nkx6.1 (Fig. 6C-D) and found that remaining insulin^{+} cells lack consistent expression of these identity markers. Similar to in *Ldb1*$^{\Delta Panc}$ pancreata, we observe maintenance of chgA expression, despite hormone loss (Supplemental Fig. 1C-D). Interestingly, in *Ldb1*$^{\Delta Endo}$ "islet clusters", we observed both hormone^{+} cells lacking TF expression (white arrows, Fig. 6C-F), and also TF^{+} cells that failed to express insulin (yellow arrows, Fig. 6C-F). These defects highlight a necessity for Ldb1 in various stages of β-cell identity and function, and perhaps within diverse β-cell populations.

Ldb1 occupies *Ngn3* and *Pdx1* control domains *in vivo* and *in vitro*

We next sought to assess Ldb1 target occupancy that may contribute to the observed impacts of Ldb1 throughout endocrine cell development. We performed a ChIP assay in Min6 β-cells for occupancy of known regulatory regions of the *Pdx1* promoter, Areas I-IV (Fig. 7A), and found that Ldb1 occupies both *Pdx1* Areas I and II (Fig. 7C). We next performed an siRNA-mediated *Ldb1* knockdown, also in Min6 cells, to assess how reduced Ldb1 levels may impact *Pdx1*Area II promoter-driven activation of a luciferase reporter. We found that upon *Ldb1* knockdown, Area II-driven luciferase activity was significantly dampened (Fig. 7D).

The *in vitro* ChIP data suggests that Ldb1 regulates *Pdx1* expression, but considering our focus on the embryonic populations we also performed ChIP experiments to assess whether Ldb1 plays a similar role in the more relevant developmental context. Despite being later than the peak of Ngn3 expression, collection of E18.5 pancreata allowed us to amass sufficient material for chromatin preparation without using non-embryonic tissue. We found that at E18.5, Ldb1 significantly occupies *Pdx1* Areas I and II, as compared to IgG control (Fig. 7E). With limited amounts of embryonic chromatin, we focused on these control domains as they are known to impart the most control during early islet development and cell specification (22, 53). Interestingly, Pdx1 occupies three evolutionary conserved upstream regions of the *Ngn3* promoter ((24), Fig. 7B, red). Considering this, and the Ngn3 and identity defects observed in our models, we investigated the occupancy of Ldb1 in these Pdx1-bound regions and found that Ldb1 also occupies regions near −4464, −4419, and −3332 bp of the *Ngn3* promoter (Fig. 7F).

Discussion

With the ongoing interest in generating β- and islet-like cells for replacement in diabetic patients (54–56), understanding the regulation of embryonic islet cell development remains exceedingly important. Our results reveal for the first time that Ldb1 is a crucial mediator of endocrine cell differentiation, with data also supporting a role for Ldb1 in postnatal islet cell function and identity (Fig. 2, 6). The Ldb1 co-regulator allows for islet progenitor (EP), nascent β- ($Pdx1^{HI}$), and eventually $hormone^{+}$ cell populations to arise (Fig. 4) and its absence imparts severe developmental and postnatal islet defects. The phenotypic similarities between $Ldb1^{\Delta Panc}$ and $Ldb1^{\Delta Endo}$ mice highlight that the role of Ldb1 in the developing pancreas appears to be largely endocrine-specific (Fig. 1 and 5). Furthermore, MPC ablation is known to impart a proportional reduction in overall pancreas size, which we did not observe here (57). This provides evidence for an interesting specificity in Ldb1 action, wherein it is required for emergence of $Ngn3^{+}$ EPs but does not impact MPC abundance as we originally hypothesized. Nonetheless, we do not observe complete loss of $Ngn3^{+}$ EPs in the Ldb1 knockouts. This could be due to incomplete *Ldb1* recombination, or perhaps remaining $Ngn3^{+}$ cells arise via the $Ngn3^{+}$ ductal pools that have recently been shown to contribute to β-cell neogenesis (58). Additionally, the maintenance of chgA staining in mutant tissues (Supplemental Fig. 1) was interesting and unexpected. These data highlight that while the cells have lost ability to express hormone, they survive and maintain some level of endocrine cell identity. ChgA was not assessed (or reported) in *Ngn3* null

animals (15), and this finding may highlight that hormone$^-$ islet cells remain despite Ngn3 loss. ChgA should be assessed in other models of hormone cell loss and dedifferentiation. Perhaps chgA maintenance reflects an opportunity to re-differentiate or recover defective cells in certain models.

We observed Ldb1 occupancy at *Pdx1* Areas I and II, as well as multiple control regions upstream of *Ngn3* (Fig. 7). Occupation of these regions required for endocrine cell development and differentiation (along with tip/trunk aberration in Fig. 3) suggests that Ldb1 is involved in endocrine progenitor lineages. It should be noted that ChIP experiments were not performed at peak Ngn3 expression, but E18.5 represents a stage where some EPs may still be present, yet more tissue is available for chromatin preparation. Other groups have also used this stage for assessment of transcription factor (Hnf6) gene occupancy in pancreatic differentiation programs (59). A potential conclusion from our ChIP data is the existence of a tightly regulated transcriptional node within specific regions of the *Ngn3* promoter, where Pdx1, Ldb1, and other transcriptional and epigenetic regulators may be occupying key *Ngn3* promoter elements in large transcriptional complexes. We postulate that this could be much like the well-studied *Pdx1* Area II domain, which is occupied and regulated by numerous factors including MafA, Pax6, and Pdx1 itself (60). In fact, mice with deletion of *Pdx1* Area II exhibit severe reduction of endocrine progenitors during development, loss of hormone$^+$ islet cells, and present with postnatal hyperglycemia (with no change in pancreas mass) similar to the $Ldb1^{\Delta Panc}$ and $Ldb1^{\Delta Endo}$ models reported here (23). Pdx1 also directly occupies and regulates *Ngn3* (24). Our data potentially incorporates Ldb1 into this network, adding another direct (Ldb1 occupying *Ngn3* domains) and indirect (Ldb1 regulating *Ngn3* through *Pdx1* regulation) contributor to the regulation of *Ngn3*. As the field continues to build on the drivers of the differentiation process, we may continue to unveil direct transcriptional regulators of this key promoter region.

The developmental pancreatic LIM-domain network continues to grow as we discover TFs and co-regulators that interact with the Ldb1-Isl1 complex, including the SSBP3 co-regulator (48) and the RNF20 and RNF40 E3 ubiquitin ligases (49). One question that remains is: which LIM transcription factor is Ldb1 interacting with to impart the (apparently Isl1-independent) EP defects exhibited in this study? Our prior study considered the LIM-HD TF Lhx1, but despite evidence for embryonic pancreas expression, we observed only postnatal β-cell functional defects in pancreas-specific Lhx1 knockouts (61). Prior work from the German lab identified the LIM-HD TFs Lmx1.1 and Lmx1.2 (also known as Lmx1a and b, respectively) to be expressed in β-cells (62). In fact, Lmx1.1, along with Cdx-3, was shown to bind and activate the *Insulin I* minienhancer (63, 64). More recent work from the Melton lab identified Lmx1b as a novel factor critical for *in vitro* endocrine cell generation (65). Their study demonstrated that core transcriptional regulatory circuits (CRCs) can be modeled by identifying superenhancer driven TFs that are interconnected in autoregulatory groups. Lmx1b was found to be a highly stage specific TF, required for the differentiation of the endocrine progenitor population. These findings present Lmx1b as a potential partner for Ldb1 in specifying endocrine cell fate in the developing pancreas.

Because of the observed roles for Ldb1 in endocrine progenitors, we sought to perform lineage tracing using a *Ngn3-Cre* driven ROSA-26-Tomato reporter model. Unfortunately,

we found that even in control animals, the reporter appeared to mark acinar cells not likely coming from a $Ngn3^+$ lineage, albeit few (data not shown). This made it difficult to interpret cells that were being misallocated due to Ldb1 loss versus cells that were positive due to potential reporter leakiness, an issue that has been described in this line with a different reporter system (42). Despite this, our data using the $Ldb1^{\Delta Endo}$ model highlight not only that Ldb1 may be involved in β-cell identity, but that diverse β-cell populations may be disrupted in the absence of Ldb1 (Fig. 6). With many studies now unveiling the existence of multiple β-cell populations (66–68), perhaps certain pools require Ldb1 for hormone expression, while others are unable to activate TF expression without Ldb1 (TF^- vs $hormone^-$ cells in Fig. 6). *Ghrelin* mRNA was unchanged at E15.5, suggesting that ε-cell differentiation is less impacted by Ldb1 loss, potentially pointing to specificity to the role of Ldb1 in EPs and subsequent islet-cell populations. Regardless, various islet-cell pools appear to require Ldb1 for proper identity and function. Considering the hypoglycemia observed in $Ldb1^{\Delta Panc}$ (Fig. 1F, right) and drastic loss of $glucagon^+$ cells in both models (Fig. 2B, 6B) future studies may also focus specifically on the role of Ldb1 in the α-cell.

Collectively, this study supports a role for Ldb1 as an islet-wide regulator of identity and function in the pancreas. The translational significance of this can be highlighted by the potential consideration of Ldb1-mediated complexes in iPSC-derived islet cell therapies aiming to use stem cells to replace β- and other islet cells for diabetic patients (69). Perhaps Ldb1 can act as a marker of successful EP differentiation and identity in these strategies, or be used as a driver, along with interacting TFs (70), of iPSCs toward EP and eventually functional hormone-secreting cells. Ldb1 requirement for emergence of islet cell types during multiple stages of pancreatic development makes it a uniquely valuable tool for our understanding and treatment of diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement:

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Abbreviations:

Cpa1	carboxypeptidase-1
chgA	chromogranin A
ChIP	chromatin immunoprecipitation

E	embryonic day
EP	endocrine progenitor
HD	homeodomain
Isl1	Islet-1
Ldb1	LIM domain binding protein-1
LMO	LIM-only
MPC	multipotent pancreatic progenitor
Ngn3	neurogenin-3
PP	pancreatic polypeptide
P	postnatal day
TF	transcription factor
TSS	transcriptional start site

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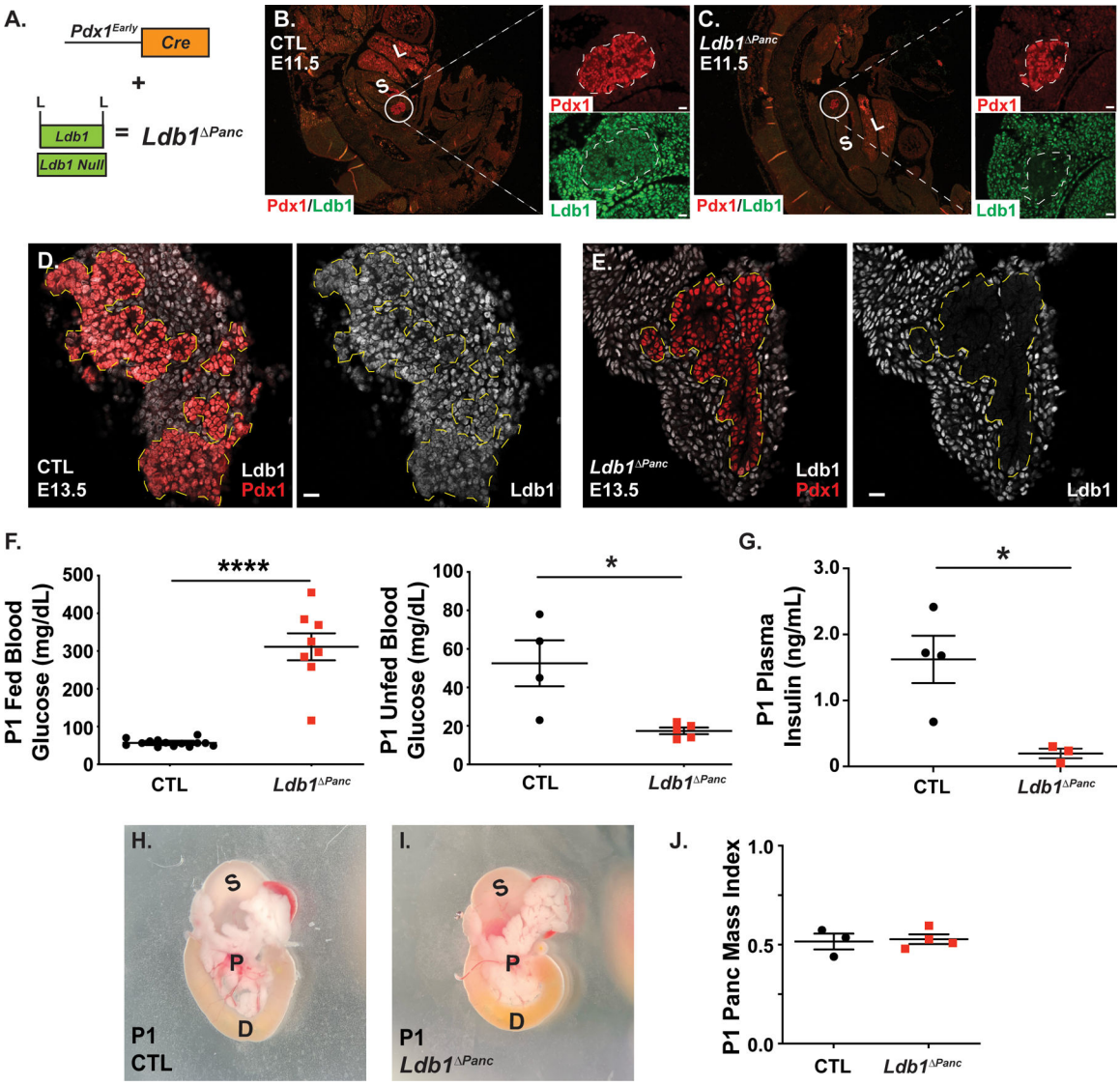

Figure 1. Ldb1 knockout by E11.5 and failure of *Ldb1*$^{\Delta Panc}$ neonates to regulate blood glucose.

A) Schematic of *Ldb1*$^{\Delta Panc}$ knockout strategy. *Ldb1*$^{F/F}$*; Pdx1-Cre* or *Ldb1*$^{+/-}$*; Pdx1-Cre* (orange) males were mated with *Ldb1* floxed/null females (green) to generate *Ldb1*$^{F/-}$; *Pdx1-Cre* embryos (*Ldb1*$^{\Delta Panc}$). B-C) Loss of Ldb1 in *Ldb1*$^{\Delta Panc}$ embryos at E11.5. Whole embryo cross-section (left) shows pancreas (dotted white line) with Pdx1 (red) and pancreas-wide Ldb1 (green). Pancreas-specific Ldb1 loss in mutants (C). S=stomach, L=liver. D-E) Loss of Ldb1 at E13.5 in *Ldb1*$^{\Delta Panc}$ embryonic pancreata as marked by Pdx1 (red) and Ldb1 (white). F) P1 blood glucose in fed (left) and unfed (right) animals, as indicated by milk spot appearance. Fed *Ldb1*$^{\Delta Panc}$ mice (red) are significantly hyperglycemic compared to controls (black), n=8–15. Unfed mutants were hypoglycemic compared to littermate controls, n=5–7. G) *Ad libitum* plasma insulin is significantly reduced in P1 *Ldb1*$^{\Delta Panc}$ mice, n=3–4. H-I) Stomach (S), pancreas (P), and duodenum (D) at P1 illustrates no overt change in pancreas size or morphology in *Ldb1*$^{\Delta Panc}$ mice. J) Pancreatic mass index was not

significantly different in *Ldb1*$^{\Delta Panc}$ as compared to littermate controls at P1, n=3. *P<0.05; ****P<0.0001. Scale bar = 20μM.

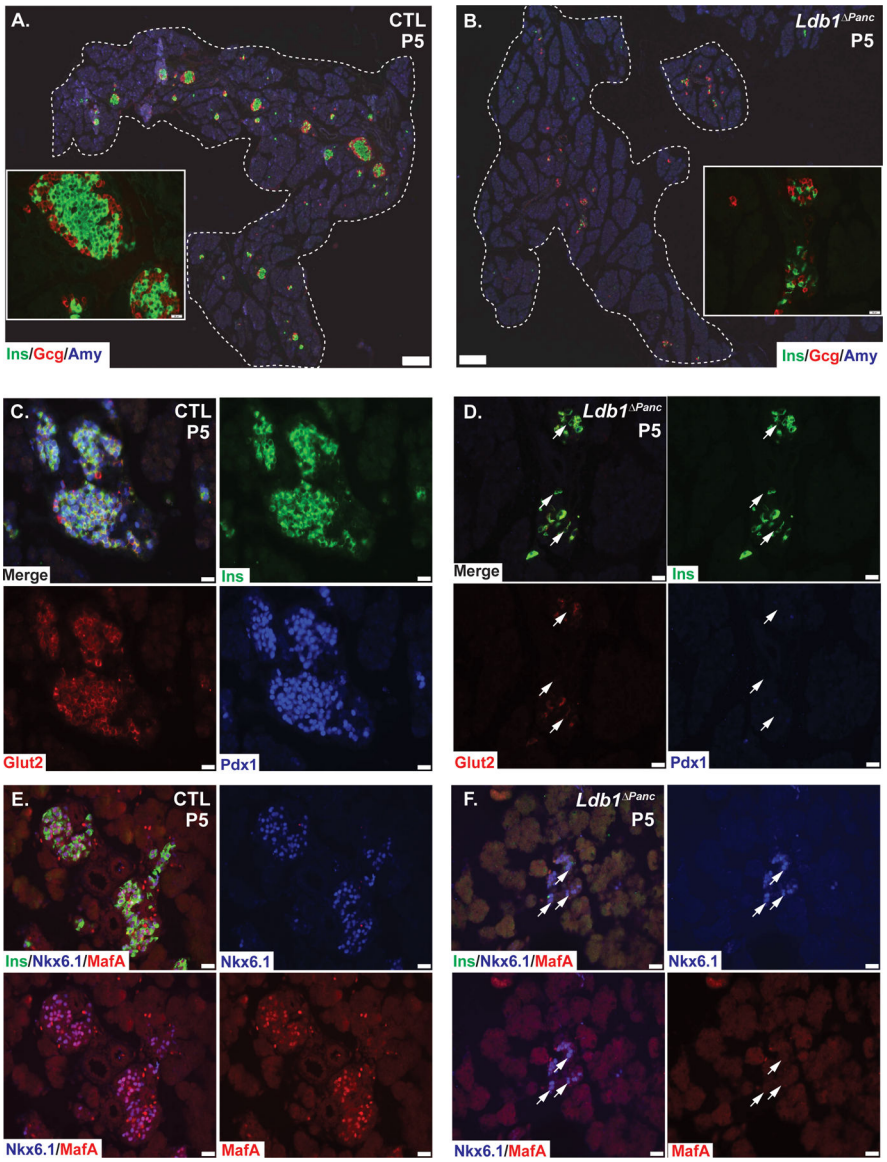

Figure 2. Lack of hormone structures and identity markers in *Ldb1*$^{\Delta Panc}$ neonates at P5.

A-B) Immunofluorescence staining for insulin (green), glucagon (red), and amylase (blue) at P5 reveals an overall lack of hormone^{+} cell clusters in *Ldb1*$^{\Delta Panc}$ model, with no observable changes in amylase expression. C) Merge shows expression of insulin (green), Glut2 (red), and Pdx1 (blue) in β-cells of control islets. D) *Ldb1*$^{\Delta Panc}$ cells exhibit Pdx1 and Glut2 dysregulation. White arrows highlight few remaining insulin^{+} cells that appear to lack Pdx1 and Glut2 expression. E-F) Expression of MafA and Nkx6.1 TFs in control and mutant mice. Bottom right control panel shows expression of MafA (red) and Nkx6.1 (blue) in β-cells. Few remaining cells in *Ldb1*$^{\Delta Panc}$ pancreata lack MafA completely but appear to maintain Nkx6.1 expression (F, white arrows, bottom left). Scale bar = 20μM.

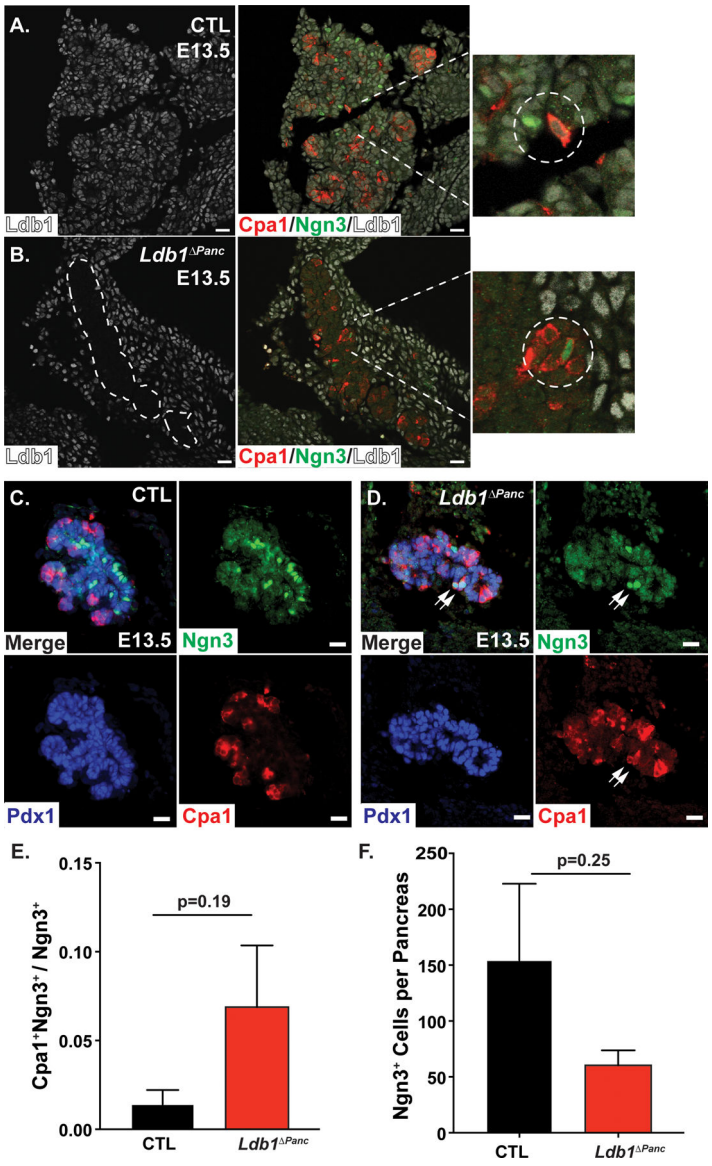

Figure 3. Apparent alteration of tip/trunk lineage domains suggests early identity defect in *Ldb1*$^{\Delta Panc}$ pancreata.

A-B) Immunofluorescence for Ldb1 (white) and Cpa1 (red, trunk marker)/Ngn3 (green, tip marker) highlights broad Ldb1 expression in control (A) and specific knockout in *Ldb1*$^{\Delta Panc}$ embryos (B, left, white outline). Double positive (Cpa^{+}/Ngn3^{+}) cell observed in Ldb1^{-} mutant progenitors (B, right inset). C-D) Immunofluorescence staining for Pdx1 (blue), Ngn3 (green), and Cpa1 (red) in control and *Ldb1*$^{\Delta Panc}$ animals at E13.5. Controls almost never exhibit co-expression of tip and trunk markers, while mutant tissues have aberrant co-expression of Ngn3 and Cpa1 (white arrowheads), highlighting potential MPC allocation defect, n=5–6. E) Quantification of co-expression represented as ratio of Cpa1^{+}/Ngn3^{+} cells over Ngn3^{+} total, n=3. F) Total Ngn3^{+} cell numbers in E13.5 pancreata highlight potential defect in endocrine progenitor appearance, n=3. Scale bar = 20μM.

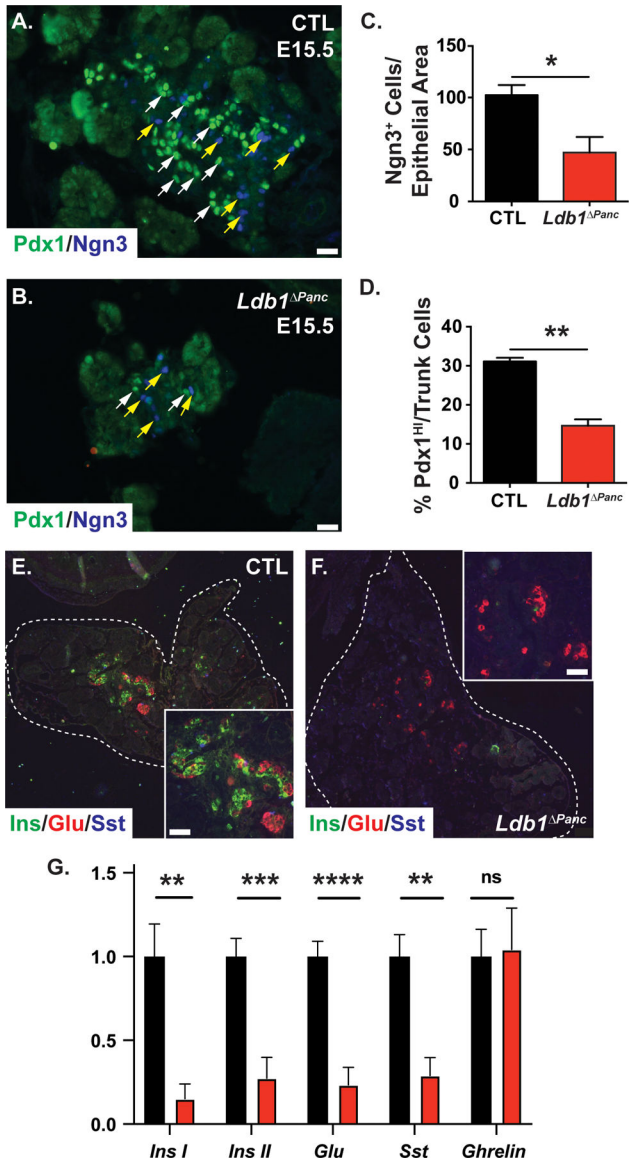

Figure 4. Ldb1 loss causes reductions in key pancreatic endocrine progenitor and early islet hormone populations at E15.5.

A-B) Immunofluorescence staining for Pdx1 (green; Pdx1^{HI} marked by white arrows) and Ngn3 (blue; yellow arrows) in control A) and *Ldb1*$^{\Delta Panc}$ tissue B). C) Quantification of Ngn3^{+} cells within the epithelial area shows reductions of this population upon Ldb1 loss, n=3. D) Pdx1^{HI} (bright green nuclei) cells are reduced in *Ldb1*$^{\Delta Panc}$. Pdx1^{HI} cells were quantified as % of Nkx6.1^{+} trunk cells, n=3. E-F) Staining for insulin (green), glucagon (red), and somatostatin (blue) in control and mutant tissue. The pancreatic domain is outlined by white dotted lines. G) Relative mRNA levels in whole E15.5 pancreas reveal reductions in *insulin I*, *insulin II*, *glucagon*, and *somatostatin* mRNA (n=14 control, 7 *Ldb1*$^{\Delta Panc}$). *P<0.05; **P<0.01, ***P<0.001, ****P<0.0001. Scale bar = 20μM.

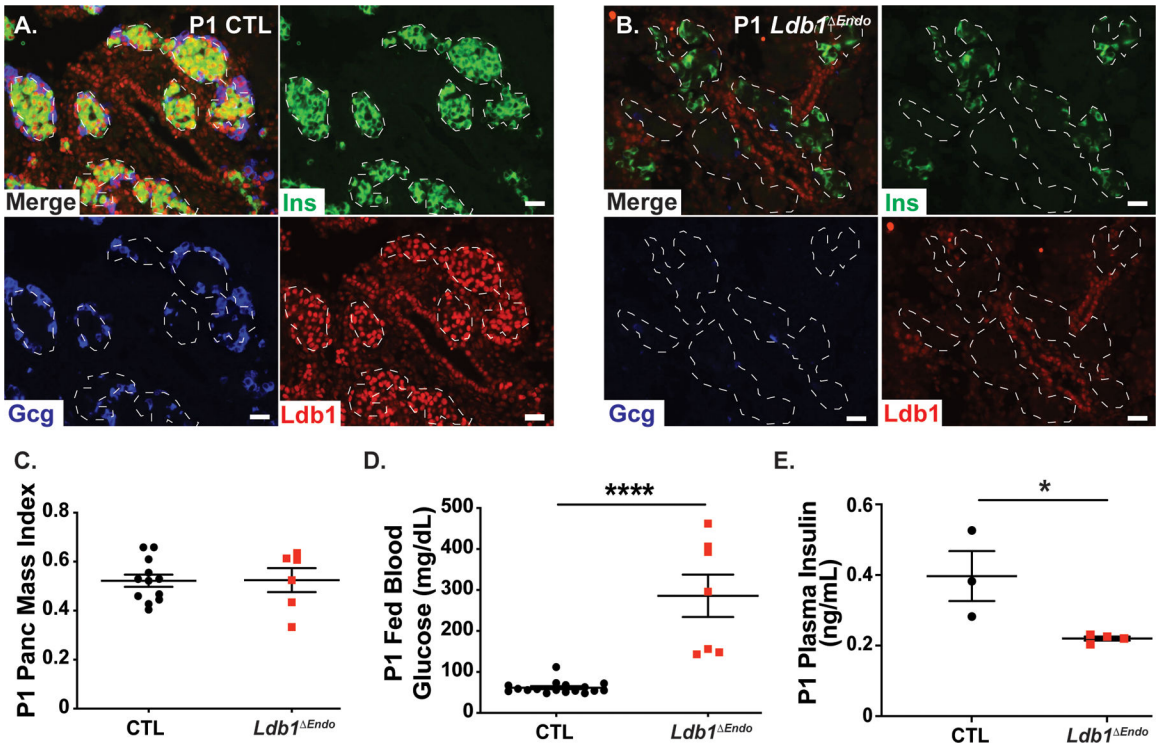

Figure 5. *Ldb1*$^{\Delta Endo}$ neonates are hyperglycemic and hypoinsulinemic, similar to *Ldb1*$^{\Delta Panc}$.

A) Ldb1 knockout specifically in endocrine clusters at P1. Staining for insulin (green), glucagon (blue), and Ldb1 (red) shows enrichment of Ldb1 in control islets and B) specific loss of Ldb1 in *Ldb1*$^{\Delta Endo}$ islet-like clusters, but not ductal or acinar cells. C) Pancreatic mass index is unchanged across genotypes, n=6–12. D) P1 blood glucose in fed pups, as indicated by presence of milk spot. *Ldb1*$^{\Delta Endo}$ pups are significantly hyperglycemic, n=7–17. E) Plasma insulin levels in *Ldb1*$^{\Delta Endo}$ mice are reduced compared to littermate controls, n=3–4. *P<0.05; ****P<0.0001. Scale bar = 20μM.

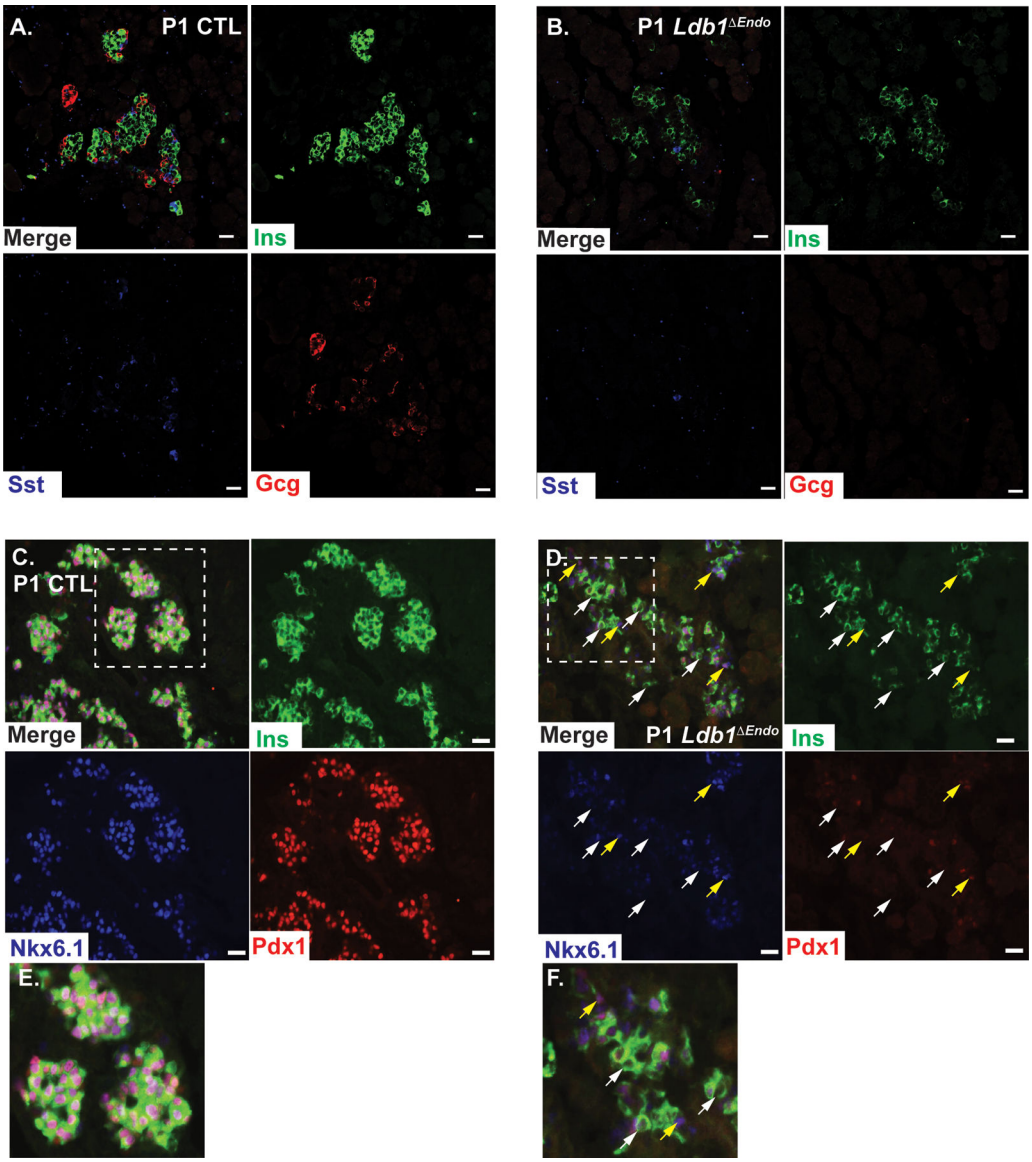

Figure 6. Drastic reduction of hormone^{+} cells and aberrant TF pattern in *Ldb1*$^{\Delta Endo}$ neonates.
A-B) Immunofluorescence for insulin (green), somatostatin (blue), and glucagon (red) in control (A) and mutant (B) neonates. *Ldb1*$^{\Delta Endo}$ mice have aberrant expression of insulin and nearly complete loss of somatostatin^{+} and glucagon^{+} cells. C-D) Immunofluorescence in control and *Ldb1*$^{\Delta Endo}$ cells for insulin (green), Nkx6.1 (blue), and Pdx1 (red). *Ldb1*$^{\Delta Endo}$ islets exhibit aberrant β-cell marker expression with insulin^{+}, TF^{-} cells (white arrows) as well as insulin^{-}, TF^{+} cells within the islet (yellow arrows). E-F) Zoomed insets of merged images highlight normal TF expression in control and irregularity in mutant tissue.

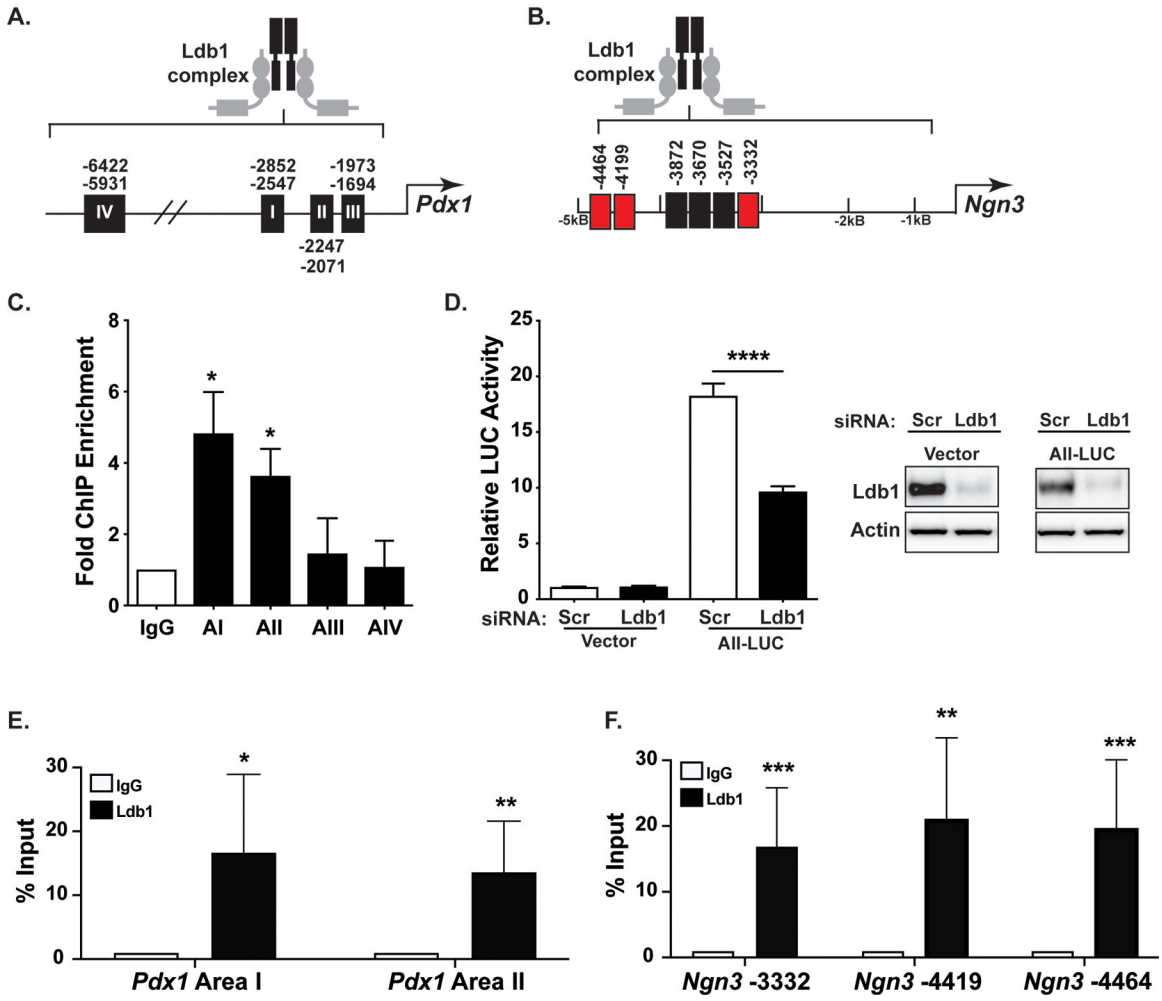

Figure 7. Ldb1 occupies key *Pdx1* and *Ngn3* regulatory domains.

A) Diagram of conserved mouse *Pdx1* promoter domains, Areas I-IV, and their respective coordinates from the TSS. Occupying complexes show Ldb1 (black) and putative interacting LIM-HD TF (grey). B) Diagram of mouse *Ngn3* promoter domains 5kB upstream of TSS, Pdx1-bound regions shown in red. C) Ldb1 ChIP in Min6 cells demonstrates significant *Pdx1* Area I-II occupation (n=3). D) Min6 knockdown and luciferase reporter assay demonstrates that Ldb1 is required for *Pdx1*-Area II activity *in vitro*, n=6. Knockdown efficiency is shown on the right by immunoblotting, compared to Actin. E-F) Embryonic pancreas Ldb1 ChIP demonstrates occupation of *Pdx1* and *Ngn3* regulatory regions of E18.5 whole pancreata, compared to IgG (1-fold), n=4–7. *P<0.05; **P<0.01; ***P<0.001, ****P<0.0001.