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Replacement of Rbpj with Rbpjl in the PTF1 complex controls the final maturation of pancreatic acinar cells

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Abstract

Background & Aims—The mature pancreatic acinar cell is dedicated to the production of very large amounts of digestive enzymes. The early stages of pancreatic development require the Rbpj-form of the trimeric transcription factor complex PTF1 (PTF1-J). As acinar development commences, Rbpjl gradually replaces Rbpj; in the mature pancreas, PTF1 contains Rbpjl (PTF1-L). We investigated whether PTF1-L controls the expression of genes that complete the final stage of acinar differentiation.

Methods—We analyzed acinar development and transcription in mice with disrupted *Rbpjl* (*$Rbpjl_{ko/ko}$* mice). We performed comprehensive analyses of the mRNA population and PTF1 target genes in pancreatic acinar cells from these and wild-type mice.

Results—In *$Rbpjl_{ko/ko}$* mice, acinar differentiation was incomplete and characterized by decreased expression (as much as 99%) of genes that encode digestive enzymes or proteins of regulated exocytosis and mitochondrial metabolism. Whereas PTF1-L bound regulatory sites of genes in normal adult pancreatic cells, the embryonic form (PTF1-J) persisted in the absence of Rbpjl and replaced PTF1-L; the extent of replacement determined gene expression levels. Loss of PTF1-L reduced expression (>2-fold) of only about 50 genes, 90% of which were direct targets of PTF1-L. The magnitude of the effects on individual digestive enzyme genes correlated with the developmental

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timing of gene activation. Absence of Rbpjl increased pancreatic expression of liver-restricted mRNAs.

Conclusions—Replacement of Rbpj by Rbpjl in the PTF1 complex drives acinar differentiation by maximizing secretory protein synthesis, stimulating mitochondrial metabolism and cytoplasmic creatine-phosphate energy stores, completing the packaging and secretory apparatus, and maintaining acinar-cell homeostasis.

Keywords

Ptf1a; transdifferentiation; ChIP-Seq; RNA-Seq

BACKGROUND & AIMS

The mammalian pancreas is principally an exocrine epithelium of acini and ducts surrounding a small amount of endocrine tissue within islets of Langerhans. The acini secrete massive amounts of hydrolytic digestive enzymes that are flushed to the intestine via a bicarbonate-producing ductal tree. The human exocrine pancreas, for example, secretes roughly one-quarter the amount of protein ingested [1, 2]. The extreme differentiation of acinar cells is dedicated to the synthesis, storage and regulated exocytosis of approximately two-dozen secretory enzymes and accessory proteins [3, 4]. Over 90% of the protein synthetic capacity of the acinar cell is committed to producing the secretory proteins [5, 4], and is supported by more ribosomes than any other cell-type in the body. Acinar cytodifferentiation creates a secretory protein factory: mammalian acinar cells are packed with rough endoplasmic reticulum, the Golgi apparatus is huge and vigorous, the apical region is dominated by protein-dense secretory granules, and numerous large active mitochondria provide copious energy.

The production of the acinar secretory proteins in the short term is constitutive; control of their secretion balances the intracellular stores and immediate use. However, Pavlov originally observed that dietary changes maintained over long periods can alter the digestive properties of pancreatic secretions [6]. More recently, dietary composition was shown to alter the transcript levels of the secretory enzyme genes for the biased digestion of protein, carbohydrate or fat [7–9]. A molecular understanding of the transcriptional mechanisms that establish and control the production of the pancreatic digestive enzymes is lacking. In this report, we show that an isoform switch in the RBP-subunit of the PTF1 transcription factor complex controls the maturation of acinar cells, including the expression pattern of the acinar digestive enzyme genes.

PTF1 (Pancreas Transcription Factor 1) is the key DNA-binding transcriptional regulator of pancreatic development and activator of the digestive enzyme genes 10, 11. PTF1 recognizes an extended bipartite DNA sequence composed of an E-box and a TC-box spaced one or two helical DNA-turns apart {CACCTG-(1 or 2 turns)-TTTCCCACG} present in the promoters of the pancreatic secretory enzyme genes 10, 12, 13. In adult acinar cells, PTF1 contains three subunits, all of which bind DNA: Ptf1a, Rbpjl, and any one of the four common (class A) basic-helix-loop-helix (bHLH) proteins 12, 14. Ptf1a is a class B bHLH protein largely restricted to the acinar pancreas in adult animals 11, 12, [15], but is required for the development of the spinal cord, retina and cerebellum [16–18] as well as the pancreas [19, 20]. Rbpjl is closely related to Rbpj [21], the widely expressed DNA-binding transcriptional mediator of the canonical Notch signaling pathway [22]. Unlike Rbpj, Rbpjl is largely confined to the pancreas and cerebral cortex [12, 23] and is not involved in Notch signaling [12, 21]. The presence of all three subunits is necessary for the acinar transcriptional activity of PTF1, and the requirement for an Rbp subunit can be satisfied by either Rbpj or Rbpjl [12]. Ptf1a and the common E protein form a canonical bHLH heterodimer and bind the E-box 10, 12. Rbpjl or Rbpj interacts with the C-terminal domain of Ptf1a and binds the TC-box, which is similar to the consensus binding sequence for Rbpj. Ptf1a

and Rbpjl co-reside at acinar specific promoters containing the bipartite site in chromatin isolated from newborn and adult pancreas [12, 24]. The form of PTF1 containing Rbpjl (PTF1-L) is the principal activator of the digestive enzyme genes through its bipartite binding-sites in their promoters.

During embryonic development, the PTF1 complex is required for the early growth and morphogenesis of the nascent pancreatic epithelium [19, 20, 24] and subsequently for the formation and differentiation of acinar cells (M. Hale, Q.L., M.A.M., & R.J.M., unpublished). During the early stage, *Rbpjl* is not expressed, and the PTF1 complex contains Rbpj (PTF1-J) rather than Rbpjl (Figure 1) [24]. At the onset of acinar development, PTF1-J binds and activates the quiescent *Rbpjl* gene promoter [24]. As Rbpjl accumulates, it gradually replaces Rbpj in the complex and on the *Rbpjl* promoter. As development proceeds, PTF1-L binds and drives the promoters of the digestive enzyme genes. In adult cells, the PTF1-complex bound to the *Rbpjl* promoter is exclusively PTF1-L, which establishes an auto-activation loop that ensures the continued production of Rbpjl. Similarly, PTF1-L binds and activates the transcriptional enhancer of the *Ptf1a* gene [25]. This dual autoregulation stabilizes PTF1-L levels and helps maintain the differentiated phenotype of pancreatic acinar cells (Figure 1).

To investigate whether the switch from Rbpj to Rbpjl is important for acinar cell differentiation, we examined the effects of inactivating the *Rbpjl* gene on pancreatic growth and acinar gene expression. Our results showed that the switch from PTF1-J to PTF1-L drives the final stage of acinar cell differentiation by enhancing mitochondrial metabolism and cytoplasmic creatine-phosphate energy stores, completing the apparatus for intracellular transport, packaging and regulated secretion, maximizing secretory protein synthesis, and suppressing the expression of hepatic genes.

RESULTS

Effects of the inactivation of *Rbpjl*

The mouse *Rbpjl* is a complex gene of twelve exons with two transcriptional start sites and two translational starts, and its genetic targeting requires special considerations (Figure 2). *Rbpjl* transcripts that begin at the downstream promoter (exon 2) do not include the first translation initiator codon and use instead an AUG in the fifth exon [23]. The long and short Rbpjl mRNAs are readily detected in RNA from adult pancreas by RT-PCR (Figure 2B). Before the presence of the short form was known, a gene knock-out attempt disrupted the long form of the Rbpjl mRNA, but left the short form intact [23]. To ensure that both forms of Rbpjl were disrupted, we replaced three central exons required for protein integrity and encoding the DNA-binding domain with the coding region for *nlacZ* (*Rbpjl* ^{ko}) (Figure 2C, D).

A complication for the inactivation of *Rbpjl* arises from the overlap of the 5' ends of *Rbpjl* and *Matn4*. *Matn4* is transcribed in the opposite direction (Figure 2A) and also expressed in the developing and mature pancreas (Figure 2E and ref. 26). The Matrilin4 protein is a widely distributed member of a family of noncollagenous extracellular matrix molecules [27], and inadvertent disruption of its expression while trying to inactivate *Rbpjl* might affect pancreatic development or function and thereby obscure the defects specific to *Rbpjl* inactivation. To avoid disrupting *Matn4* transcriptional control sequences, we limited the deletion to 1.3-kb of *Rbpjl/Matn4* 5-kb upstream of the closest *Matn4* transcriptional start (Figure 2A), which has little cross-species sequence conservation other than the *Rbpjl* exons. The *Matn4* mRNA levels, measured by quantitative RT-PCR, were unaltered in embryonic (E17.5) and mature pancreas from mice homozygous for the *nlacZ* replacement (*Rbpjl*$^{ko/ko}$) relative to levels in heterozygous normal pancreases (Figure 2E).

Development of the pancreas in *Rbpjl*-knockout mice appeared normal, except that the pancreatic mass of $Rbpjl^{ko/ko}$ mice at 8 weeks and 8 months of age was one-third less than normal (Figure 3). The DNA and RNA contents of heterozygous $Rbpjl^{+/ko}$ and homozygous $Rbpjl^{ko/ko}$ pancreases at birth were not different ($p = 0.57$; Figure 3); therefore, the number of cells and the RNA content per cell (mostly rRNA) were approximately equivalent. Moreover, tissue and cellular morphologies of the mutant pancreas viewed by light and electron microscopies were indistinguishable from normal (data not shown), except that the acinar cells of the mutant mice were 35% smaller ($p=0.0001$), which is sufficient to account for the difference in total pancreatic weight. The reduced size of the Rbpjl-deficient acinar cells appears related to the greatly reduced production of the secretory enzymes (see below).

Inactivation of *Rbpjl* had no discernible effect on the endocrine pancreas: islet hormone mRNA levels, islet tissue organization, fasting glucose levels and the results of glucose tolerance tests were comparable to normal (data not shown). Thus, defects were restricted to the exocrine compartment of the pancreas, and no other defects were detected throughout the embryo or adult.

Defects of acinar gene expression

To determine whether the absence of Rbpjl affected the expression of known target genes of the PTF1 complex, we compared the levels of the mRNAs for 21 digestive enzymes from normal and $Rbpjl^{ko/ko}$ pancreases at a late stage in development (E17.5) when the PTF1-L version predominates [24]. All of the acinar secretory protein genes have recognizable PTF1-complex binding sequences in their promoters that are conserved among mammals (Table S1); of the four sites assayed individually by immunoprecipitation of pancreatic chromatin, all have resident Ptf1a (Figure 5A). Therefore, all of the acinar genes for digestive enzymes are likely direct PTF1-targets. The level of each of the mRNAs (except Cpa1 mRNA) was decreased between 50 and 98% in the knockout pancreas (Figure 4A). For example, all four trypsin mRNAs measured decreased to 4% or less of their level in normal pancreas. The magnitude of the effect correlates with the timing of transcriptional activation of their genes during pancreatic development, and appears related to the switch of Rbpjl for Rbpj in PTF1 (see the Discussion). Moreover, because the amount of ribosomal RNA per cell remained constant, rRNA biosynthesis is unlinked from Rbpjl.

To determine whether the absence of Rbpjl affected the occupancy of a PTF1 complex on target genes, we measured the relative amounts of Ptf1a residing on the promoters of four representative secretory enzyme genes and the *Rbpjl* gene in chromatin from adult pancreas by conventional quantitative chromatin immunoprecipitation (ChIP). Ptf1a was still present, but at reduced levels, on all five promoters (Figure 5A). The partial retention of Ptf1a suggests that Rbpj might partly compensate for the absence of Rbpjl by forming the PTF1-J complex normally present only during early pancreatic development. Indeed, whereas Rbpj is undetectable on these promoters (no enrichment by ChIP) in wild type[12] or $Rbpjl^{+/ko}$ pancreas, it was present in homozygous *Rbpjl*-deficient pancreas (Figure 5B). Moreover, the relative increase of Rbpj at the four digestive enzyme promoters (Ctrb>Ela1>Ela2a>Try4; Figure 5B) correlated with the level of maintenance of the individual mRNAs (53%>18%>9%>2%, respectively; Figure 4A). Because the amount of Ptf1a on these promoters decreased, it appears that Rbpj does not maintain promoter occupancy by the PTF1 complex as well as Rbpjl does, which is consistent with the decreased levels of mRNAs for these digestive enzymes.

The effects of Rbpjl inactivation are highly selective

To determine whether the effects of *Rbpjl*-inactivation were restricted to the control of the digestive enzyme genes, we compared the mRNA populations of the normal and $Rbpjl_{ko/ko}$ E17.5 pancreases by RNA-Seq. At this developmental stage, acinar cells are largely, but not

fully differentiated, and defects of development as well as maturation should be detectable. We detected 4,062 genes expressed at an estimated 3 mRNA molecules per average cell or greater. The RNA transcripts for only 53 of these genes decreased 2-fold or more in the *Rbpjl* mutant pancreas (Table S2). This analysis confirmed the Q-rtPCR results for the greatly diminished secretory enzyme mRNAs (Figure 4B). Most other affected mRNAs encode proteins for the intracellular transport and packaging of the secretory enzymes, their regulated secretion, and mitochondrial metabolism (Table 1). If these proteins are important for maximal acinar function and then we would expect their mRNAs to be maintained or increased in adult pancreas relative to the less differentiated E17.5 pancreas. This is indeed the case for all but one of the secretory enzyme genes as well as for 19 of the 23 non-secretory enzymes in Table 1.

To determine whether the affected genes are direct targets of PTF1-L, we searched for sites bound by Ptf1a and Rbpjl in chromatin from normal adult pancreas using Chip-Seq. We identified 6,165 genomic sites with bound Rbpjl (T.D., C.S. and R.J.M., in preparation) using parameters that limited the predicted false discovery rate to less than 10^{-5} (see Supporting Procedures). Of the total Rbpjl-bound sites, the great majority (>87%) had coincident binding of Ptf1a, indicating the presence of the PTF1-L complex. Thus, the transcriptional action of Rbpjl in the adult pancreas appears to be mediated nearly exclusively through PTF1-L. Indeed, 48 of the 53 genes affected by *Rbpjl* inactivation had co-resident Ptf1a and Rbpjl associated with a recognizable bipartite PTF1-binding site within 10-kb, including those for the metabolic enzymes and the packaging components (Figure 6). Because the 48 genes with strongly reduced expression account for such a small fraction of genes with co-resident Ptf1a and Rbpjl, the specific effect of Rbpjl *vs*. Rbpj on acinar gene transcription is highly limited. Nonetheless, the overall impact of Rbpjl on the level of acinar differentiation is large.

Because the PTF1 complex appears to sit near the top of a regulatory hierarchy for acinar development, we searched the differential transcriptome data for mRNAs of DNA-binding transcription factors that in the $Rbpjl^{ko/ko}$ versus the normal pancreas at E17.5. No transcription factor mRNA decreased more than 2-fold, although the mRNAs of two (Onecut1/Hnf6 and Prox1) increased more than 3-fold. It appears that the onset of *Rbpjl* expression during acinar development does not induce additional transcriptional regulators.

Liver-specific genes are induced in *Rbpjl*-deficient pancreas

In contrast to the decreases for some genes, the expression of eighty-five genes increased 4-fold or more in the absence of Rbpjl. Of the fifteen genes most highly induced by the absence of Rbpjl, eight encode proteins characteristic of the hepatic secretory profile (Figure 4B), such as albumin (20-fold), alpha-fetoprotein (30-fold), and several apolipoproteins (10- to 43-fold). The induction of hepatic genes is not accompanied by increases in liver-restricted transcription factors.

The induction of liver genes was, in part, a stochastic process. Because the global analysis of mRNA by RNA-Seq was performed on pools of RNAs from three $Rbpjl^{ko/ko}$ or three $Rbpjl^{+/+}$ pancreases, the results reflect the average of the three samples. When the three RNA samples were assayed individually for a subset of four liver mRNAs by Q-rtPCR, only one or two of the pancreases had an increase of any one liver mRNA and the relative levels of induction varied greatly (Figure S1). It is unlikely that the PTF1-L complex directly represses liver genes, because none of the induced liver genes has an associated peak of Ptf1a in normal pancreatic chromatin (data not shown), and there is no evidence that the PTF1 complexes can act as repressors. These observations are consistent with a general lessening of repression of liver genes, but offer no clues to a specific mechanism.

DISCUSSION

We have shown that the final state of acinar differentiation depends on Rbpjl through a selected subset of genes that are bound and activated by the Rbpjl-form of the PTF1 transcription factor complex. The PTF1-L complex binds approximately 5,360 genomic sites in adult pancreatic chromatin associated with 2,633 mRNA-coding genes. In the absence of Rbpjl, Rbpj can substitute partially for Rbpjl, and the mRNAs of only 53 genes with significant expression decrease more than 2-fold. The effects of the Rbpjl-knockout are progressively greater for genes that are activated later during acinar development, and so the affected genes normally complete the final stage of acinar differentiation. Most encode the secretory enzymes and proteins required for their efficient synthesis, storage and secretion. The Rbpjl knockout also releases some liver-specific gene expression, which indicates that an additional function of the PTF1-L form is to maintain acinar identity rigorously.

Several lines of evidence indicate that gene-control by *Rbpjl* is direct and mediated by Rbpjl within the trimeric PTF1-L complex rather than through independent functions of Rbpjl. **First**, all but five of the affected genes have co-resident Ptf1a and Rbpjl in normal adult chromatin. **Second**, the bHLH-binding E-box and the Rbp-binding TC-box as well as proper spacing [12] are necessary for the activity of the bipartite PTF1-site in transgenic mice [24 25]. **Third**, mutational inactivation of *Rbpjl* decreased the amount of Ptf1a bound to PTF1-sites and the level of residual expression correlated with the amount of Rbpj replacement in the PTF1 complex. However, we cannot rule out a PTF1-independent function for Rbpjl with other genes and other contexts.

Control by Rbpjl is selective for the late stage of acinar development

The accumulation of the various secretory proteins or their mRNAs during normal pancreatic development is not coordinate [28, 29]. Cpa1 appears first, and the trypsin family members are among the last, approximately four days later. Based on the known temporal accumulation patterns of ten secretory enzyme mRNAs [28], the magnitude of the effect of the Rbpjl knockout on individual mRNAs correlates with the timing of transcriptional activation of their genes during pancreatic development (see Figure 4A). For example, Cpa1 mRNA was unaffected, whereas mRNAs for all four trypsins were among the most highly reduced and the mRNAs for other secretory proteins (black bars in Figure 4A) were decreased by intermediate amounts roughly correlated with the timing of their developmental appearance. We propose that the timing of the activation of digestive enzyme genes during development and the effects of the absence of Rbpjl on these genes are due to the differential ability of PTF1-J and PTF1-L to activate the promoters of these genes. The genes least affected by the absence of Rbpjl are those activated by PTF1-J prior to the appearance of Rbpjl and the formation of the PTF1-L complex. The last secretory protein genes activated depend almost entirely on the L-form of PTF1.

Although PTF1-L is bound to several thousand acinar genes, genome-wide RNA-Seq analysis showed that only 53 genes decreased 2-fold or greater in Rbpjl-deficient pancreas. The affected genes are largely limited to digestive enzymes and a few additional components of highly differentiated acini and do not include additional transcription factors. The absence of effects on transcription factor genes indicates that Rbpjl does not control acinar differentiation through other transcriptional regulators, but rather directly on genes for the differentiated functions of acinar cells.

We conclude that the Rbpj-form of PTF1 is sufficient to initiate acinar development and drive early differentiation, and relatively few Rbpjl-dependent genes complete the process of acinar differentiation. These few genes encode key components of mitochondrial energy metabolism, RNA and protein synthesis, and the packaging, intracellular transport and regulated exocytosis

of secretory proteins (Table 1). Rbpjl enhances the expression of five strategic metabolic enzymes of mitochondria that provide sources of carbon and nitrogen as well as energy production and distribution. Tdh, Bdn2, Gls2 and Gatm are highly regulated gatekeepers of linked mitochondrial metabolic pathways (Figure 6D) and are localized to the same matrix-side compartment of the inner mitochondrial membrane 30–32 (with the exception of Gatm, which is on the outer aspect of the inner mitochondrial membrane 33, 34). We propose that the induction of these five key enzymes during the final stage of differentiation by the Rbpjl form of PTF1 establishes a highly active metabolic state that provides the extraordinary amounts of substrates and energy required by mature acinar cells for RNA and protein synthesis, intracellular transport, and exocytosis.

Several additional proteins encoded by genes that require PTF1-L for maximal expression are part of the secretory packaging machinery. Syncollin, Cuzd1 and Zg16 are components of the zymogen granule membrane and critical to exocytosis and the proper initiation of the zymogen activation cascade [35, 36, 37]. Tmed11 is a member of a family of proteins that transports protein cargo from the endoplasmic reticulum to the Golgi 38, 39. Dmbt1 is a Golgi cargo receptor needed for the trafficking to a stimulus-releasable pool 40, [41]. Uroplakin (Upk1a) helps define the apical plasmalemma endocytic domain for recycling secretory granule membrane [42]. All are largely restricted to the pancreas, stomach and intestine and their induction would be expected to enhance the secretory phenotype. As expected for key components of the secretory apparatus, Sync, Dmbt1 Cuzd1 and Zg16 increase further in the adult and are among the forty highest copy pancreatic mRNAs.

The genes for these mitochondrial enzymes and trafficking components have resident Ptf1a and Rbpjl at PTF1-binding sequences in chromatin from adult mouse pancreas (Table 1). Thus, Rbpjl appears to act on these genes directly through the PTF1-L complex. Overall, the induction of these genes appears to optimize energy production and distribution, enhance protein synthesis and modification, complete the packaging apparatus for the secretory proteins, and add components that regulate exocytosis.

Rbpjl suppresses the hepatic phenotype in acinar cells

The absence of Rbpjl leads to the induction of liver-specific genes in the pancreas. This effect appears to be indirect – there is no evidence that PTF1-L acts as a repressor of hepatic genes, and Ptf1a binding was not detected in the vicinity of those genes by Chip-Seq. Nonetheless, the function of Rbpjl for the enhancement and maintenance of the acinar phenotype includes the suppression of liver gene expression. The liver and the ventral pancreas are resolved from a region of the foregut endoderm by dynamic interplay among TGFβ, BMP and FGF signaling pathways [43]. The similarity between pancreas and liver development extends to the shared use of several phenotype-determining transcription factors [44, 45]. Indeed, transdifferentiation between pancreatic acinar and hepatic phenotypes can be induced *in vivo* and *in vitro* [46, 47]. Therefore, it is not surprising that the absence of a differentiation factor such as Rbpjl could lead to a mixed pancreas/liver phenotype.

Although the PTF1-J complex is ancient [12], PTF1-L is a recent innovation, since Rbpjl first appears in the vertebrate lineage and is directly responsible for the extreme phenotype of pancreatic acini. Our molecular, genetic and genomic analyses explain the mechanism for completing the modern version of this exocrine gland.

EXPERIMENTAL PROCEDURES

Generation of *Rbpjl*^{lacZ} (*Rbpjl*^{ko}) knockout mice

Details of the knockout construct for Rbpjl are in Supporting Information. All animal experiments were in accordance with the guidelines of the University of Texas Southwestern Institutional Animal Care and Use Committee or the Vanderbilt University Animal Care Program.

RNA isolation, quantitative rtPCR, and transcriptome analysis by RNA-Seq

Total RNA was isolated from the pancreas of individual E17.5 day embryos using Trizol (Invitrogen). After genotyping of the embryos, the RNA preparations of three wild-type (C57Bl/6, Rbpjl$^{+/+}$) and three knockout (C57Bl/6, Rbpjl$^{ko/ko}$) littermates were used for further study. Complementary-DNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and oligo(dT) primer. For Q-rtPCR experiments, RNA from individual animals was assayed. Samples of cDNA derived from 2 ng of total cellular RNA template were amplified in 20 μl reactions with gene-specific primers (primer list is available upon request). The amount of cDNA was quantified with SYBR Green using an Applied Biosystems 7500 Fast instrument and normalized to the level of β-actin mRNA.

Comprehensive analysis of the mRNA population in E17.5 day wild type or knockout pancreas was performed by RNA-Seq [48] using 10 μg of total RNA pooled from the three wild-type pancreas RNA preparations or pooled from the three knockout pancreas RNA preparations, according to the Illumina RNA-Seq protocol (Illumina Inc., Hayward, CA). Details of the sequencing parameters and the bioinformatics are provided in Supporting Information.

Chromatin Immunoprecipitation

Immunoprecipitation of chromatin from mouse pancreas was performed as described previously [12]. The sequences of the primer pairs used to detect specific gene promoter regions are available upon request. The amount of precipitated DNA was quantified with SYBR Green using the Applied Biosystems Prism 7700. The extent of enrichment was calculated from the amount of target promoter DNA relative to the amount of the 28S region of the large ribosomal RNA gene in the immunoprecipitated chromatin versus the starting chromatin. ChIP-Seq was performed as described in protocols from the manufacturer (Illumina) with only minor modifications. Further details and bioinformatic parameters are provided in Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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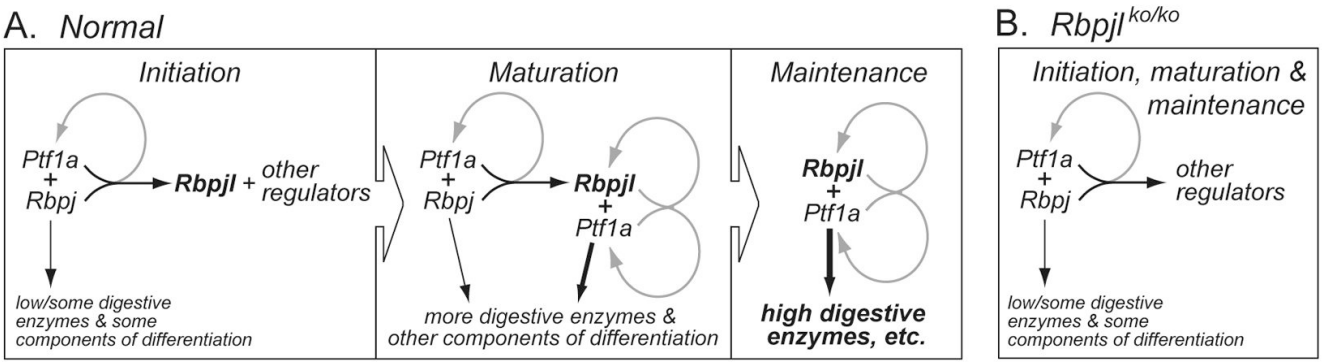

Figure 1.

PTF1 regulation of acinar development. **A.** ***Initiation***: The acinar lineage is initiated prior to the induction of *Rbpjl*. Ptf1a, Rbpj and a common E-protein form PTF1-J, which auto-activates *Ptf1a* transcription. ***Maturation***: The early PTF1-J target-genes include *Rbpjl* as well as some of the secretory enzyme genes. The Rbpjl form of the PTF1-complex, PTF1-L, begins to accumulate and is more effective than PTF1-J on most of the secretory enzyme genes. PTF1-L establishes dual auto-activation loops that sustain high transcription rates of *Ptf1a* and *Rbpjl*. ***Maintenance***: In the mature acinar pancreas, PTF1-L is the sole form, is sustained at high levels by autoregulation, and maintains a maximal acinar phenotype. **B**. In the absence of Rbpjl, PTF1-J persists, but is less effective on genes for secretory proteins and other select acinar proteins.

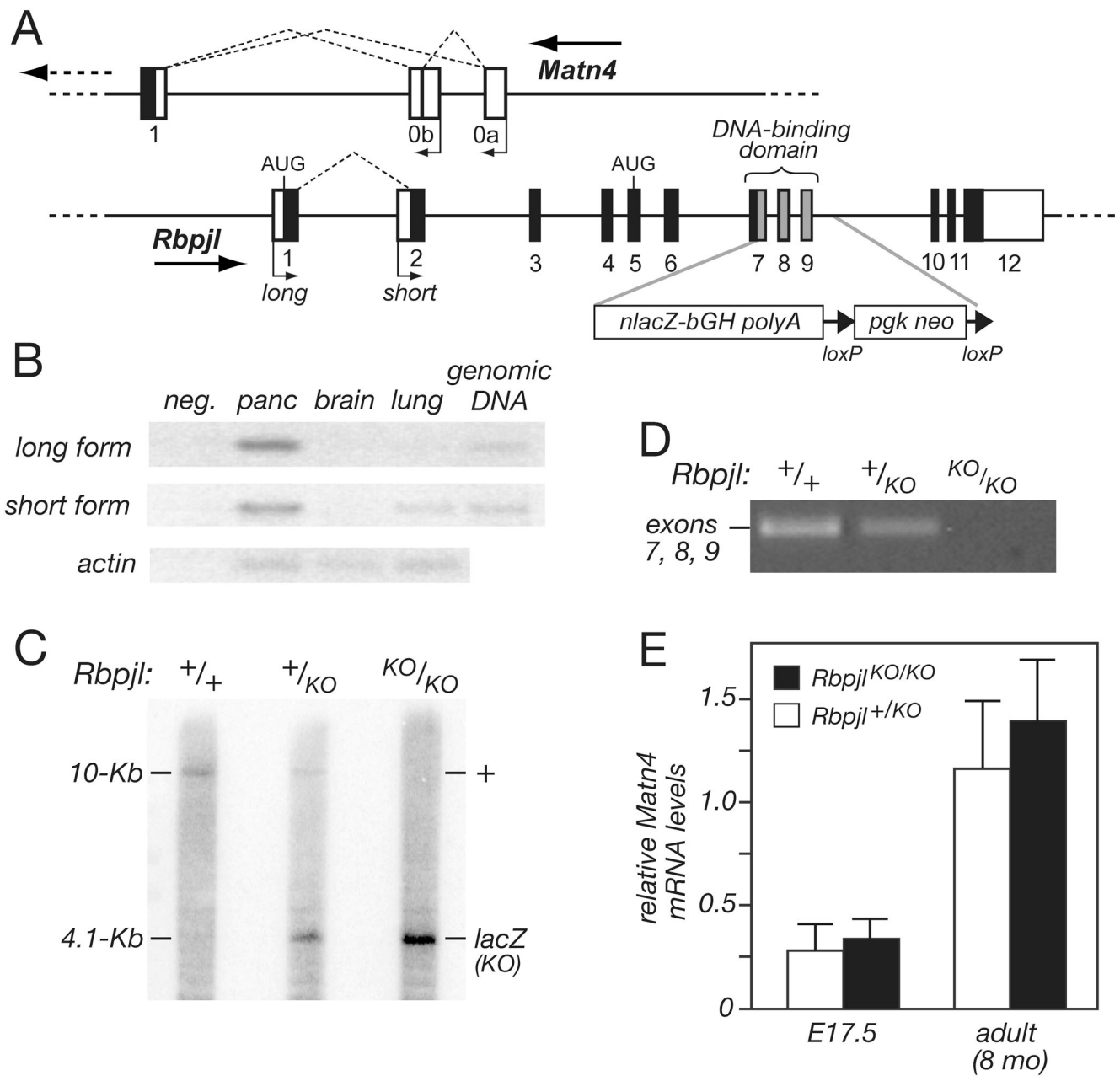

Figure 2.

Engineered inactivation of *Rbpjl*. **A**. The structure and disruption of the mouse *Rbpjl* gene. Black and grey boxes indicate coding exons; white, 5' and 3' untranslated regions. A central *Rbpjl* gene region (*grey* exons) was exchanged for an *nlacZ* linked to a bGH 3' untranslated region and polyA signal. **B**. rtPCR detection of the long or short forms of Rbpjl mRNA. **C**. Southern hybridization with NcoI-cleaved genomic DNA showed the loss of wild type (10-Kb) and gain of *lacZ* (4.1-Kb) alleles. **D**. The deletion of *Rbpjl* exons 7, 8 and 9 was confirmed by PCR amplification analysis. **E**. Q-rtPCR analysis of Matrilin4 mRNA from adult and E17.5 pancreases. The levels of Matn4 mRNA are relative to wild type adult pancreas levels.

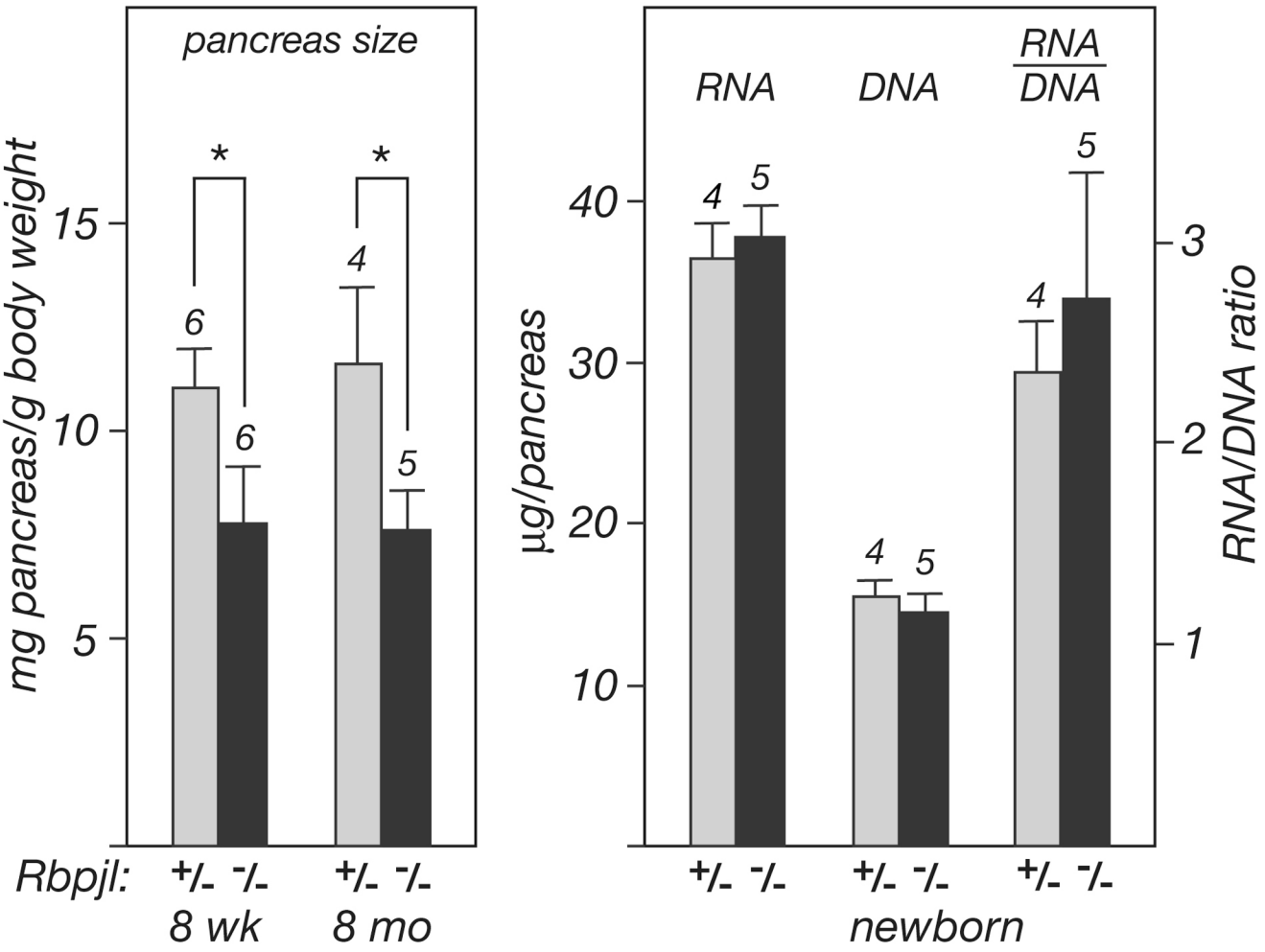

Figure 3.

Effects of *Rbpjl* inactivation. **A**. The pancreases of *Rbpjl*$^{ko/ko}$ mice at 8-weeks or 8-months of age were one-third smaller than those of age-matched *Rbpjl*$^{+/ko}$ mice. **B**. RNA and DNA content of newborn pancreases. The numbers of mice analyzed for each genotype are indicated (*p<0.05; **p<0.01). Error bars are SEMs.

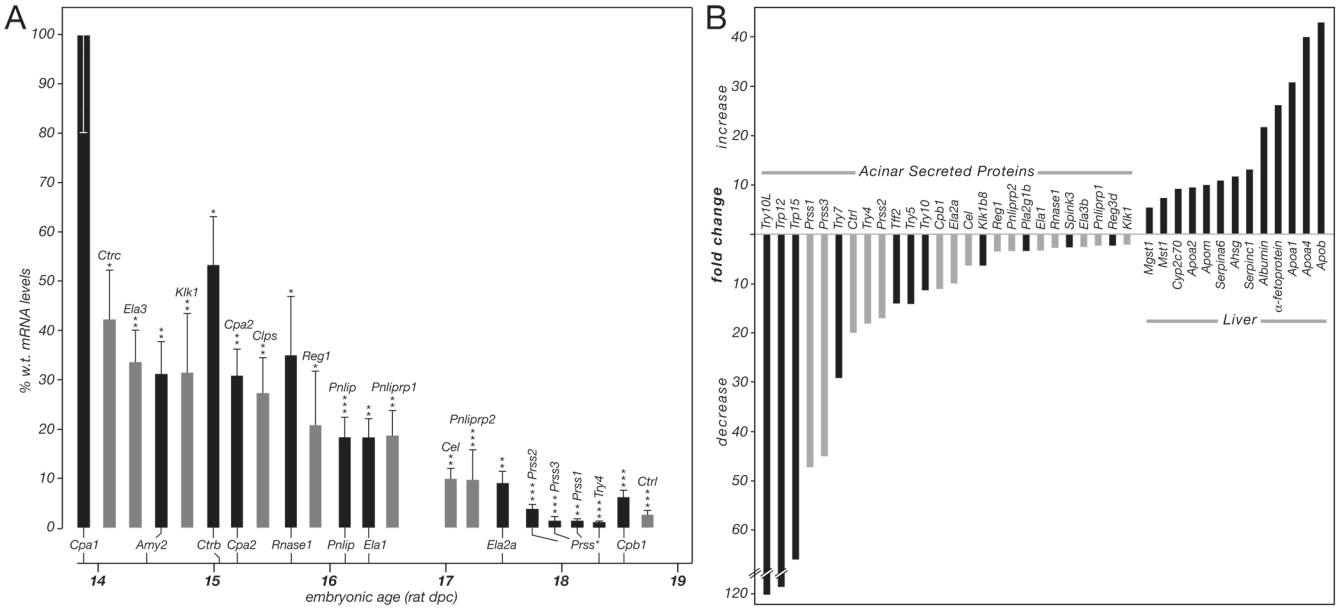

Figure 4.

Changes in pancreatic gene expression. **A**. *Rbpjl* inactivation decreases the levels of the mRNAs encoding the acinar digestive enzymes. The mRNA levels for E17.5 pancreas were quantified by Q-rtPCR and are expressed relative to the level of the mRNA in normal E17.5 pancreas (*P<0.05; **p<0.01; ***p<0.001). Error bars are SDs. The *black* bars are positioned at the known developmental age of the appearance of the mRNA in embryonic rat pancreas [28]. The midpoint of the first decade of mRNA accumulation was taken as a measure of the time of appearance. The remaining mRNAs (*grey* bars) are placed relative to the known mRNAs according to their levels in *Rbpjl*$^{ko/ko}$ pancreas, in prediction of the timing of their appearance. **B**. Results of the genome-wide analysis of pancreatic mRNAs for *Rbpjl*$^{ko/ko}$ and *Rbpjl*$^{+/+}$ embryos at E17.5 by RNA-Seq. *Grey* bars indicate mRNAs measured by Q-rtPCR as well in panel A.

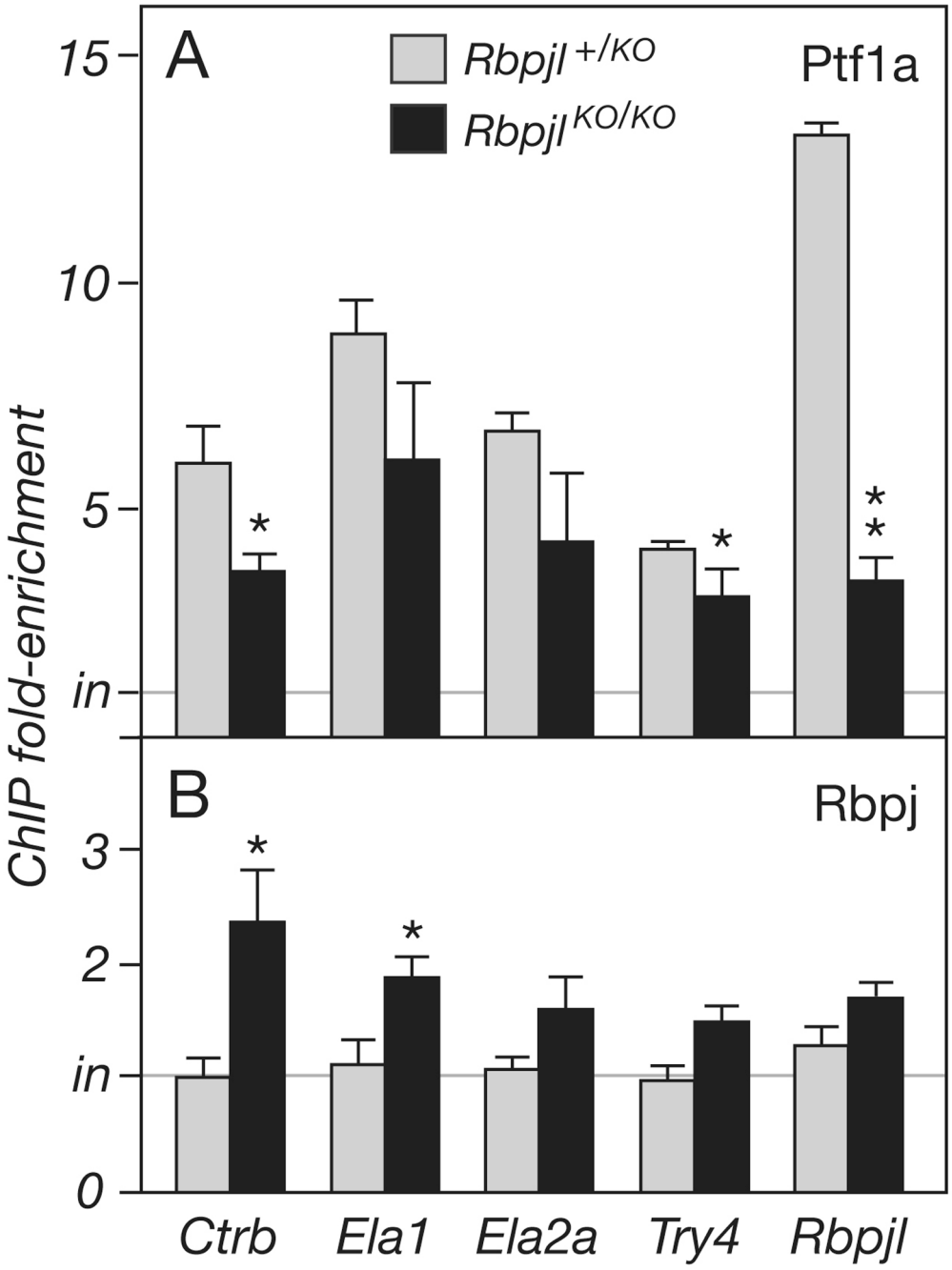

Figure 5.

The absence of Rbpjl partly depletes PTF1a from target promoters (**A**) and leads to detectable Rbpj (**B**) on the same promoters in adult pancreatic chromatin (*P<0.05, **P<0.01). *in* (input) is the result using DNA from chromatin without immunoprecipitation and represents no enrichment. The amounts of Ptf1a and Rbpj on these promoters were unchanged between *Rbpjl*$^{+/+}$ and *Rbpjl*$^{+/ko}$ mice, and neither Rbpjl nor β-galactosidase was detected on chromatin from *Rbpjl*$^{ko/ko}$ mice (data not shown). Fisher's method for combining the probabilities of multiple tests of a hypothesis [49] provides a *p* value <0.001 that Ptf1a occupancy does not decrease (panel A) and a *p* value <0.01 that Rbpj occupancy does not increase (panel B) on PTF1 target genes in *Rbpjl*$^{ko/ko}$ chromatin. Error bars are SEMs.

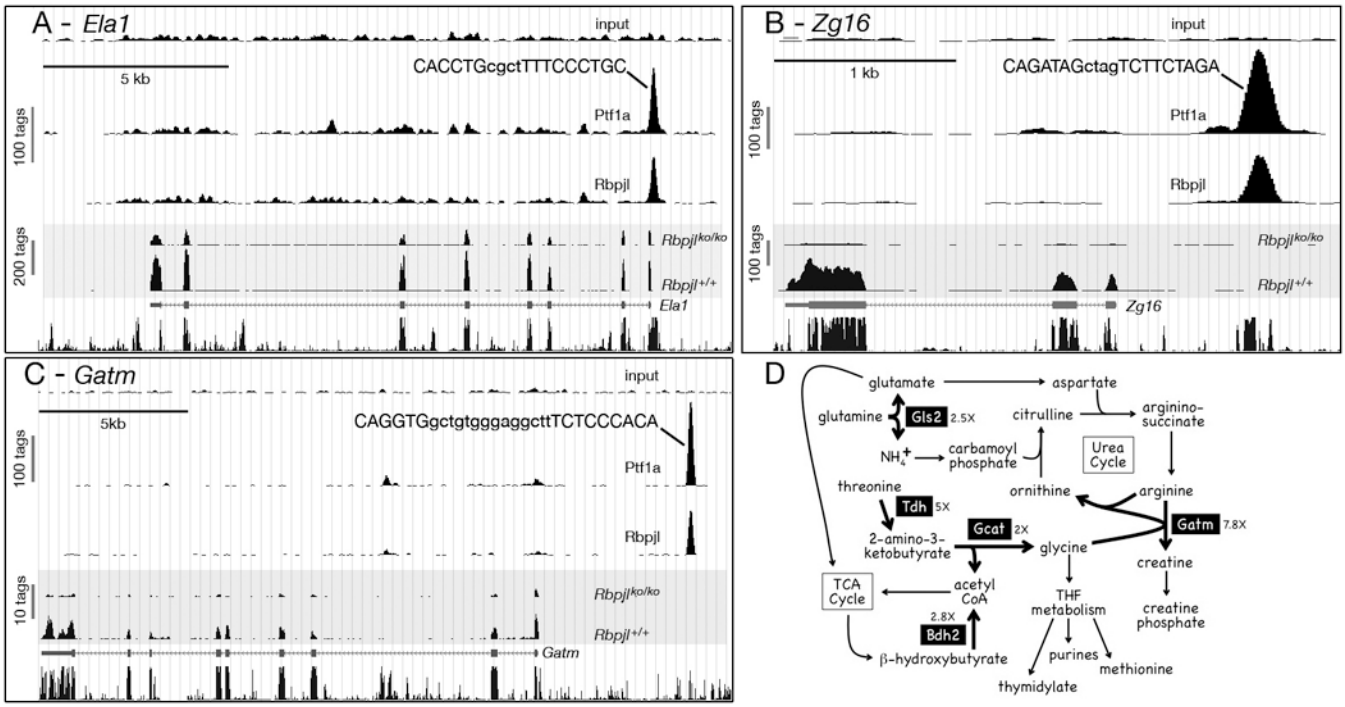

Figure 6.

Chip-Seq and RNA-Seq results for examples of (**A**) a secretory enzyme (*Ela1*), (**B**) a secretory packaging protein (*Zg16*), and (**C**) a metabolic enzyme (*Gatm*). Shown for each, from top to bottom: ChIP-Seq for input DNA, Ptf1a-binding, and Rbpjl-binding; RNA-Seq (*grey highlight*) for E17.5d pancreas from $Rbpjl^{+/+}$ and $Rbpjl^{ko/ko}$ embryos; the exon organization for the Ref-Seq mRNA; and the mammalian conservation from BLAT [50]. The sequences of the PTF1 binding site within the peaks of Ptf1a and Rbpjl binding are given (capital letters, E- and TC-boxes). Scale bars indicate the peak height for the sequence tags. **D**. Mitochondrial metabolic pathways linking the functions of Gls2, Gatm, Tdh, Gcat and Bdh2.

Table 1

Non-secretory enzyme genes affected by *Rbpjl* gene inactivation.

Mitochondrial metabolism		**mRNA fold-change**		**ChIP peaks**[a]			
		Decline for E17.5 Rbpjl$^{-/-}$	**Increase from E17.5 to adult**	**Position**[b]	**Ptf1a**	**Rbpjl**	**Input**
Gatm	Glycine amidinotransferase	8.5	5.1	5’ dist	524	175	4
Tdh	Threonine dehydrogenase	5.1	1.6	in1, 3’	118, 241	59, 155	8, 28
Gls2	Mitochondrial glutaminase	3.1	5.1	5’ dist, i9	121, 278	69, 122	5, 6
Bdh2	β-hydroxybutyrate dehydrogenase	2.6	0.01*	in3	221	56	13
Gcat	Mitochondrial threonine & acetyl-CoA catabolism	2.0	1.3	in1	519	193	14
Secretory protein modification, packaging, transport and regulated exocytosis							
Zg16	Zymogen granule packaging protein	17	25	5’ dist	474	186	10
Cuzd1	Integral zymogen granule membrane protein	10	44	ex1	386	139	8
Tmed11	Pancreas protein for intracellular transport	6.9	0.7	in1	45	6^{ns}	2
Sync	Zymogen granule maturation and secretion	6.3	11	5’ dist	52	16	4
Lfng	O-fucosyl 3-beta-N-acetylglucosaminyltransferase	2.5	0.6	5’ dist	782	405	13
Dmbt1	Muclin; Golgi cargo receptor for protein trafficking	2.2	167	5’ prox	118	43	7
Upk1a	Uroplakin; recycling secretory granule membrane	2.2	0.04*	3’	51	16	8
Derl3	Degradation of unfolded secretory proteins in ER	2.0	4.8	3’	281	141	12
Miscellaneous							
Gal	Neuropeptide	53	2.4	5’ dist	106	42	8
Tff2	Secreted trefoil factor 2	12	33	5’ prox	134	56	14
Zp3	Secreted glycoprotein, unknown somatic cell function	11	0*	in5	50	11	0
Slc39a5	Zinc ion transporter	3.1	3.7	in7	255	126	7
Spink3	Secretory pancreatic trypsin inhibitor	2.8	2.9	in3	147	81	7
Fbfbp1	Fibroblast growth factor binding protein	2.6	0.02*	3’	56	35	12
Vtn	Vitronectin; promotes cell adhesion	2.3	2.9	ex1	72	29	6
Serinc3	Serine transporter; phosphatidylserine biosynthesis	2.2	3.2	in8, in9	53, 88	26, 33	3, 5
Pkiag	cAMP-dependent protein kinase inhibitor gamma	2.2	0.6	5’ dist	88	33	5

Mitochondrial metabolism		mRNA fold-change		ChIP peaks[a]			
		Decline for E17.5 Rbpjl$^{-/-}$	Increase from E17.5 to adult	Position[b]	Ptf1a	Rbpjl	Input
Slc6a9	Glycine transporter	2.0	5.1	5' dist, in2	291, 571	207, 190	14, 14

The fold-changes in mRNA levels were calculated as the ratio of the rpkm values from the RNA-Seq data for *Rbpjl*$^{+/+}$ and *Rbpjl*$^{ko/ko}$ pancreases. Rpkm is the cumulative number of sequence reads (tags) for all of the exons of an mRNA divided by the length in kilobases of the mRNA and normalized to one million total reads. To limit consideration to genes with significant expression, those with mRNA levels below 10 rpkm (approximately 3 mRNAs per cell) were not included in this compilation. Ptf1a ChIP peak values are the total number of sequence tags in the peak(s) associated with the cognate gene in a ChIP-Seq experiment. Four genes (Hist1h2af, Tmem54, Tct9, Agr2) with more than two-fold decreased expression are without significant Ptf1a or Rbpjl binding and are not included. The total number of sequence tags (millions) for the ChIP-Seq experiments are Ptf1a, 5.8; Rbpjl, 4.9; and input, 5.0. (see also Table S2)

[a] only the major peaks of Ptf1a and Rbpjl binding are listed for each gene.

[b] *5' prox*, within 1-kb of the transcriptional start site; *5' dist*, farther than 1-kb but closer than 10-kb; *exN* or *inN*, in the N$_{th}$ exon or intron of the gene, respectively. All *3'* positions were farther than 1-kb from the 3' exon.

[*] genes that significantly decreased expression between E17.5 and adult. Because the increase in digestive enzyme mRNAs between E17.5 and adult decreases the apparent level of other mRNAs, the levels of all adult mRNAs were normalized according to the average ratio (Adult *vs*. E17.5 = 0.21 +/− 0.08) of mRNAs for four 'housekeeping' genes: *Tbp*, *Hprt*, *Sfrs9*, and *Rbpl23*.