Conditional ablation of Notch signaling in pancreatic development

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The role of the Notch signaling members Notch1, Notch2 and Rbpj in exocrine pancreatic development is not well defined. We therefore analyzed conditional pancreas-specific Rbpj and combined Notch1/Notch2 knockout mice using Ptf1a+/Cre(ex1) mice crossed with floxed Rbpj or Notch1/Notch2 mice. Mice were analyzed at different embryonic stages for pancreatic exocrine and endocrine development. The absence of Rbpj in pancreatic progenitor cells impaired exocrine pancreas development up to embryonic day 18.5 and led to premature differentiation of pancreatic progenitors into endocrine cells. In Rbpj-deficient pancreata, amylase-expressing acini and islets formed during late embryonic and postnatal development, suggesting an essential role of Rbpj in early but not late development. Contrary to this severe phenotype, the comitant inactivation of Notch1 and Notch2 only moderately disturbed the proliferation of pancreatic epithelial cells during early embryonic development, and did not inhibit pancreatic development. Our results show that, in contrast to Rbpj, Notch1 and Notch2 are not essential for pancreatogenesis. These data favor a Notch-independent role of Rbpj in the development of the exocrine pancreas. Furthermore, our findings suggest that in late stages of pancreatic development exocrine cell differentiation and maintenance are independent of Rbpj.

**KEY WORDS:** Notch, Rbpj, Conditional knockout mice, Pancreas, Development

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**INTRODUCTION**

The Notch signaling pathway is a key regulator of developmental processes during organogenesis. Loss-of-function studies have proposed that Notch signaling regulates self-renewal leading to depletion of pancreatic progenitor cells and accelerated differentiation of endocrine cells (Apelqvist et al., 1999; Fujikura et al., 2006; Fujikura et al., 2007; Jensen et al., 2000). While these studies have provided evidence for an important role of Notch signaling in endocrine development, the dependence of the exocrine compartment on specific Notch signaling members is not well understood. Recently, RBPJκ, the transcriptional mediator of Notch signaling, was found to be a binding partner of PTF1A in the PTF1 complex, suggesting a Notch-independent function during pancreatic development (Beres et al., 2006; Masui et al., 2007). Moreover, because of the existence of multiple receptors, ligands and target genes, and because of the embryonic lethality of mice null for Notch signaling components, the precise role of individual Notch signaling components in early and late pancreatic organogenesis is not well defined. During pancreatic organogenesis, Notch1 and Notch2 expression has been described in the pancreatic epithelium, whereas Notch3 and Notch4 are expressed in mesenchymal and endothelial cells (Lammert et al., 2000).

In order to clarify the role of the epithelially expressed receptors Notch1 and Notch2 versus the abrogation of RBPJκ signaling, we analyzed conditional Notch1/Notch2 double-knockout and Rbpj knockout mice by using Ptf1a+/Cre(ex1) mice for targeting pancreatic progenitor and exocrine cells.

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**MATERIALS AND METHODS**

**Generation of Rbpjf/f mice**

In order to generate a conditional gene replacement vector for Rbpj, several genomic fragments of Rbpj were isolated from a λ-DASHII phage library (Stratagene) and subcloned into pBluescript SK+ (Stratagene). A neomycin resistance cassette, flanked by two loxP sites, was inserted into intron 7, and a single loxP site was integrated into intron 5. A herpes simplex virus thymidine kinase (HSV-1k) cassette was cloned at the 5’ end of the gene-targeting construct (Fig. 1A). The Not1-linearized vector was transfected into murine 129/SvJ ES cells, where it recombined with the host genome. The homologous recombination event occurred at a frequency of 1:602 and was verified by PCR and Southern blot analysis. The floxed neo-resistance cassette was removed by transient transfection of a vector expressing the Cre-recombinase. Blastocyst injection and germline transmission of the mutant allele were done as described previously (Tanigaki et al., 2002). Genotyping of mice was performed on DNA isolated from tail biopsies using a PCR kit (Qiagen). For the detection of floxed (0.55 kb fragment) and wild-type (0.5 kb) alleles of Rbpj, PCR amplification (1 minute at 94°C, 30 seconds at 58°C and 30 seconds at 72°C, for 40 cycles) was carried out using primers 5’-AGT TTA GGC TTT CCA AAA GGC-3’ (forward) and 5’-GTA TTG CTA AGA ACT TGT TGC-3’ (reverse). All mice were housed in pathogen-free conditions. All mouse protocols were approved by the Centre of Animals Research, the Faculty of Medicine, Technical University of Munich.

**X-gal staining**

β-gal activity was determined on whole-mount preparations as described previously (Kawaguchi et al., 2002).

**BrdU labeling**

In vivo pulse labeling with 5-bromo-2-deoxyuridine (BrdU) was used to mark newly synthesized DNA. BrdU (20 mmol/l, 5 ml/kg body weight) was injected intraperitoneally into pregnant mice 2 hours before sacrifice.

**Histology and immunohistochemistry**

Dissected tissues were fixed in ice-cold 4% paraformaldehyde, paraffin-embedded and cut into 2-3 μm sections. Immunohistochemistry was performed using the following primary antibodies: rabbit anti-PTF1A (1:500, kind gift from Raymond J. Macdonald, University of Texas, USA); rabbit anti-PDX1 (1:10,000, kind gift from C. V. Wright, Vanderbilt University Medical School).
Center, Nashville, USA); guinea pig anti-insulin (1:1000, Linco); rabbit anti-glucagon (1:1000, Linco); guinea pig anti-glucagon (1:500, Linco); rabbit anti-β-galactosidase (1:500, ICN); rabbit anti-somatostatin (1:1000, ICN); rabbit anti-pancreatic polypeptide (1:500, BioTrend); rabbit anti-amylase (1:1000, Sigma) rabbit anti-phosphohistone H3 (1:500, Upstate); rabbit anti-carboxypeptidase A (1:500, BioTrend); mouse anti-CK19 (1:500, Developmental Studies Hybridoma Bank (DSHB), University of Iowa); rabbit anti-HES1 (1:100, kind gift from T. Sudo, Toray Industries, Tokyo, Japan); rabbit anti-cyclin B1 (1:500, Millipore); rat anti-BrDU (1:250, Serotec); mouse anti-Neurogenin (1:500, DSHB). For immunoperoxidase detection, Vectastain ABC kit (Vector Labs) was used according to the manufacturer’s instructions. For double immunofluorescence staining, the primary antibodies were followed by incubation with secondary antibodies conjugated with fluorescent Alexa 488 or Alex 568 (Molecular Probes). Sections were mounted with Vectashield mounting medium (Vector Laboratories) and examined using an Axiosvert 200M (Zeiss) fluorescent inverse microscope equipped with the Axiosvision version 4.2 software (Zeiss). The number of islets was calculated, with the definition of an islet being a group of β cells surrounded by α cells. For morphometric analyses, the pancreatic buds were immunostained with anti-PDX1 and analyzed using the AxioVision Image analysis software (Zeiss). To calculate the number of PHH3- and neurogenin 3-positive cells, the pancreatic buds were dissected from 5- to 6-month-old mice, and whole pancreatic buds from three control and three knockout embryos were cut into 3 μm serial sections. Every fifth section was stained and the number of PHH3+, neurogenin 3+ and insulin+ cells were counted and calculated relative to the whole area of PDX1+ pancreatic epithelium in every section.

**RESULTS**

Generation of pancreas-specific Rbpj and Notch1/Notch2 knockout mice

*Ptf1a+/Cre(ex1)* mice were generated using a knock-in approach replacing exon 1 with Cre recombinase (Nakahai et al., 2007). For analysis of Cre recombinase expression, *Ptf1a+/Cre(ex1)* mice were crossed to *Rosa26RlacZ* (R26R) reporter mice (Soriano, 1999). In pancreata of newborn mice, Cre-induced recombination measured by X-gal staining occurred as expected from previous studies (Kawaguchi et al., 2002) (data not shown). To abrogate Notch receptor signaling, an approach generating either *Rbpj* or *Notch1/Notch2* double-knockout mice was chosen. For simplicity, *Ptf1a+/Cre(ex1);Rbpj*+ and *Ptf1a+/Cre(ex1);Notch1*+/−;Notch2*+/− mice will be termed *RbpjKO* and *Notch1/2KO* mice, respectively. To generate pancreas-specific, *Rbpj* knockout mice, *loxP* sites were inserted flanking exons 6 and 7 of *Rbpj* in embryonic stem cells (ES) by homologous recombination (Fig. 1A). ES cell clones with a *floxed* (*Rbpj*) locus were used to generate chimeric mice. The mutant mouse line (*Rbpj*KO) was established through germline transmission. Cre-recombinase-mediated deletion of exon 6 and 7 of *Rbpj* resulted in a mutant RBP-Jκ protein lacking a functional DNA-binding domain. By crossing *Rbpj*KO mice with a Nestin-Cre deleter line, we were not able to obtain *Rbpj*-deficient newborns, and this provides strong evidence for a functionally null *Rbpj* transcript after Cre-induced recombination (data not shown). For pancreas-specific targeting, we next crossed *Rbpj*KO mice with *Ptf1a+/Cre(ex1)*. *Ptf1a+/Cre(ex1) KO* mediated deletion of the *Rbpj* gene was verified by Southern blot analysis with DNA from different tissues of newborn *Ptf1a+/Cre(ex1);Rbpj*KO mice (data not shown). For conditional knockout of Notch1 and Notch2, previously described *Notch1*KO and *Notch2*KO mice were used (Radtke et al., 1999; Schouwey et al., 2006). As we did not find qualitative defects in pancreatic organogenesis, nor major abnormalities in the unstimulated adult pancreata of conditional *Notch1*, *Notch2* or combined *Notch1/Notch2* knockout mice over an observation period of 18 months (Siveke et al., 2008) (data not shown), combined *Notch1/2KO* were chosen for the analysis of pancreatic development. In *Notch1* and *Notch2* single receptor knockouts, as well as in double-knockout pancreata, *Notch1* and *Notch2* protein and transcripts were decreased to less than 10%, as analyzed by western blot and RT-PCR (data not shown). Both, *Rbpj* and *Notch1/Notch2* knockout lines were tested for the possibility of mosaic Cre-induced recombination using *Rosa26R* reporter mice as a surrogate for recombination-induced deletion of *Rbpj* or *Notch1/Notch2*, respectively. Using X-gal staining, we did not observe X-gal-negative exocrine cells in adult pancreata (Fig. 1G-I). Regarding recombination in the endocrine compartment, we found approximately 50% of endocrine cells to be X-gal positive, a recombination pattern that was very similar to that observed in *Ptf1a+/Cre(ex1);Rosa26RlacZ* pancreata (Fig. 5M-U).

Although heterozygous *Rbpj*+/− and homozygous *Notch1KO*, *Notch2KO* and *Notch1/2KO* mice showed no gross abnormalities and developed normally, *RbpjKO* mice survived only until 4-5 days postpartum. Although moderate signs of growth retardation were observable at birth (Fig. 1C), the early death was caused by insufficient postnatal growth with impaired milk digestion. We were able to raise some *RbpjKO* mice to adulthood by feeding them with pancreatic enzyme-enriched animal food (data not shown). Examination of the *RbpjKO;R26R* mice at day 1 postpartum (dpp) revealed a small and severely altered pancreas (Fig. 1F-I). In the duodenal part of the mutant pancreas, weakly branched ducts were observable (Fig. 1F, arrowhead), whereas the splenic part of the pancreas showed no branching (Fig. 1F, arrows). Histological examination demonstrated a lack of acinar tissue with large duct-like structures being present in the splenic and duodenal portion of the pancreas (Fig. 1I, blue). Interestingly, *Notch1/2KO* mice did not reveal striking abnormalities in pancreatic tissue organization or cell lineage distribution, suggesting a non-essential role for *Notch1* and *Notch2* during pancreatic development. However, the mutant pancreas was noted to be slightly smaller than that of wild type when analyzed at 1 dpp (Fig. 1E, H). To further clarify the role of ablated Notch signaling, early stages of pancreatogenesis were investigated.
Early pancreatic development in Rbpj- and Notch1/2-deficient pancreata

To analyze pancreatic development at defined stages of pancreatic organogenesis, we investigated pancreatic bud development at embryonic day 10 (E10) to E13.5 by immunohistochemistry and X-gal staining. The pancreatic buds of E13.5 Rbpj+/–;R26R and Notch1/2KO;R26R embryos displayed the typical branching of the pancreatic epithelium. The Notch1/2KO buds appeared smaller and less branched than in control littermates (Fig. 2A,B,D). By contrast, RbpjKO;R26R embryos revealed a significantly reduced epithelial mass with weakly branched structures in both buds (Fig. 2C,D), suggesting that Rbpj is essential for the expansion of the pancreatic epithelium.

As Hes1 is one of the target genes of Rbpj-dependent Notch signaling activation, we analyzed HES1 expression at E12.5. At this stage, HES1 protein was broadly detected within the nuclei of PDX1+ epithelial cells, as well as in PDX1+ mesenchymal elements (Fig. 2E). Compared with Rbpj+/–;R26R littermate controls, RbpjKO and Notch1/2KO mice showed reduced, although not absent, HES1 expression in PDX1+ pancreatic cells, suggesting efficient ablation of Notch signaling (Fig. 2F-G). As the premature differentiation of pancreatic progenitor to endocrine cells has been suggested previously as a possible cause for the reduction of pancreatic epithelium in Rbpj-deficient buds (Fujikura et al., 2006), pancreatic sections at E11.5 were stained for X-gal and glucagon expression. In Rbpj+/–;R26R embryos, glucagon-expressing cells formed small clusters peripheral to and dispersed within the dorsal bud (Fig. 2H, brown). Similar to Rbpj+/–;R26R mice, Notch1/2KO;R26R and Rbpj+/–;R26R pups. Arrowhead in F indicates weakly branched ducts in pancreatic rudiment. neo’, neomycin-resistance gene; HSV-tk, herpes simplex virus thymidine kinase gene; d, duodenum; I, liver; p, pancreas; sp, spleen; st, stomach. Scale bar: 100 μm.

As expression of the transcription factor neurogenin 3 (Ngn3) is a prerequisite for endocrine lineage development, E13.5 pancreata were analyzed for expression of Ngn3. Consistent with previous results, we found decreased numbers of Ngn3+ cells in RbpjKO mice at this stage (Fig. 2M,N), when compared with Rbpj+/–;R26R embryos (Fig. 2K,N), suggesting an early commitment of these cells to endocrine cell lineages. These cells were found within and peripheral from the ventral and dorsal buds (Fig. 2J, brown).

Regarding the differentiation of insulin+ β cells per PDX1+ area in RbpjKO pancreata (Fig. 2Q,R).
endocrine cells in Notch1/Notch2-deficient pancreata, no significant reduction of NGN3+ cells (Fig. 2L,N) and no related β cell increase were notable at E13.5 (Fig. 2P,R). The reduced branching and epithelial mass in the Notch1/2KO and RbpjKO embryos was accompanied by a decrease in the number of proliferating cells in pancreatic epithelium, as detected by phospho-histone H3 (PHH3) and PDX1 double-immunostaining (Fig. 2S-U). While the relative number of the PHH3+ cells to PDX1+ cell area in Notch1/2KO buds was reduced by 25% in comparison with control mice (Rbpj+/–;R26R; Fig. 2S,T,V), the relative number in RbpjKO buds was decreased to 40% (Fig. 2U,V).
The microdissection of early glucagon+ cells not expressing recombinase activity, we analyzed the recombination of the floxed epithelial cells of pancreatic buds from (A) Microdissection of pancreatic epithelial cells. (B) Note that in the dorsal bud by immunostaining for glucagon at E12.5. (C) Schematic maps of the floxed Notch1 (C) and Notch2 (D) locus before and after Cre-induced recombination. The position and polarity of the primers used for recombination are represented by P1, P2 and P3 for Notch1, and P4, P5, P6 and P7 for Notch2 (red arrowheads). DNA was isolated from pancreatic epithelial cells of two Notch2 knockout embryos (lane 1 and lane 2). As a control, DNA from heterozygous Notch1/2+/- mice was used (lane 3).

In contrast to the severe defects in pancreatic development observed in RbpjKO embryos, Notch1/2KO embryos showed only a marginal loss of the pancreatic mass, without defects in the development of the exocrine and endocrine compartments. One obvious explanation would be low expression, or inefficient recombination activity, of Cre recombinase during early pancreatic development, and, consequently, an inefficient inactivation of both Notch1 and Notch2 alleles. To determine the efficiency of Cre recombinase activity, we analyzed the recombination of the floxed Notch1 and Notch2 alleles by PCR analysis of microdissected epithelial cells of pancreatic buds from Notch1/2KO embryos at E12.5. The microdissection of early glucagon+ cells not expressing Ptf1a was avoided by immunostaining the embryonic sections for glucagon. The epithelial cells from stained sections were isolated using a P.A.L.M. Microdissection system (Fig. 3A,B). The PCR was performed with DNA from two knockout embryos using specific primers for floxed and deleted Notch1 and Notch2 alleles, with DNA from heterozygous Notch1/2+/- embryos serving as a control for floxed and recombinated alleles. For the detection of recombined DNA events, 4000 cells were used for each PCR reaction with a determined sensitivity to detect recombinated alleles from DNA of about 200 cells. As shown in Fig. 3C,D, we could not detect any floxed Notch1 and Notch2 fragments in the DNA of double-knockout epithelial cells. Thus, Notch1 and Notch2 alleles were deleted in more than 95% of epithelial cells of Notch1/2KO pancreatic buds.

Appearance of acinar cells during late embryonic development in Rbpj-deficient pancreas
Exocrine cell differentiation in Rbpj+/- embryos was similar to that of RbpjWT and Notch1/2WT embryos, suggesting that heterozygosity for Rbpj and/or Ptf1a has no profound effect on exocrine lineage development. At E14.5, well-defined exocrine acini were observable in all of the littermate controls (Fig. 4A). However, in RbpjKO embryos neither amylase nor carboxypeptidase A expression was detectable at E14.5 (Fig. 4C, data not shown). In contrast to this, Notch1/2KO pancreata revealed amylase+ cells, although the acinar compartment appeared smaller than in littermate controls (Fig. 4B). At E18.5, the exocrine pancreas showed no morphological differences in Notch1/2KO, when compared to control littermates (Fig. 4D,E). In RbpjKO at this stage, we surprisingly detected a few amylase+ acini in the duodenal part and amylase+ duct-like structures in the splenic portion of the rudimentary pancreas (Fig. 4FJ). These splenic amylase+ duct-like structures contained amylase+ cells (Fig. 4K, arrows), whereas most duodenal duct-like structures did not express amylase (Fig. 4L, arrow). In RbpjKO pups, the cells of both spleenic and duodenal duct-like structures showed positive staining with CK19, a marker of differentiated ductal cells (Fig. 4I, black). These cells were also positive for X-gal staining, suggesting that all of them derived from Rbpj-deficient cells (Fig. 4I, arrows). In Notch1/2KO mice, ductal cells were CK19+ and similar in appearance to in control littermates (Fig. 4G,H, arrows).

In RbpjKO mice at E18.5, co-immunostaining for PDX1 and amylase showed that the majority of amylase+ cells were also PDX1+, and were mitotically active, as determined by BrdU labeling (Fig. 4M,N). Because a functional PTF1 complex is required for the expression of acinar genes, such as amylase, we determined the expression of PTF1A in RbpjKO pancreata. Here, we detected PTF1A+ cells surrounded by stromal cells outside the main duct and in the duodenal part of the rudimentary pancreas (Fig. 4O, arrows). To determine whether the Rbpj gene was actually deleted in amylase-expressing cells in the mutant pancreas, pancreatic sections from RbpjKO newborns were co-stained for X-gal and amylase, demonstrating the Cre-induced recombination of amylase+ cells (Fig. 4P). In addition, PCR analysis of DNA isolated from amylase+ cells by microdissection confirmed that the Rbpj gene was deleted in acinar cells from RbpjKO newborns (Fig. 4Q).

Endocrine cell development in Rbpj- and Notch1/Notch2-deficient pancreas
Most mature endocrine cells appeared after E14 in both Notch1/2KO and RbpjKO embryos, similar to in littermate controls. At E18.5, we could detect all endocrine cell lines, glucagon-producing α cells, insulin-containing β cells, somatostatin+ δ cells, and pancreatic polypeptide+ (PP) cells in both Notch1/2KO and Rbpj+/- embryos. In RbpjKO mice, these cells were detectable in the rudimentary pancreas within the tubular duct wall and in the protruding formations of the pancreatic tubule (Fig. 5A-L).

At E18.5, most endocrine cells of the Rbpj+/- control embryos aggregated with α cells starting to organize around core structures of β cells (Fig. 5P, arrowhead). Similar to control embryos, endocrine cells in both Notch1/2KO and RbpjKO embryos also started to aggregate; however, the number of formed islets was
less than in control embryos (Fig. 5V). Regarding the morphological appearance of the formed islets, the endocrine epithelium in Notch1/2KO embryos, and more prominently in RbpjKO embryos, had a disturbed appearance. In most of the endocrine cell formations, α cells were not organized around β cells, and the morphology of these islet-like structures appeared to be long rather than circular like in the control mice (Fig. 5P,Q,R). In adult pancreata of Notch1/2KO mice, however, the islets appeared normal and were indistinguishable from wild-type controls (data not shown).

Because Ptf1a<sup>+/-;Cre(ex1);R26R</sup> mice show mosaic Cre-induced recombination in the endocrine compartment (data not shown), we sought to analyze whether this mosaicism was also present in the different knockout lines. Co-staining of islets from Rbpj<sup>+/–;R26R</sup>, Notch1/2KO;R26R and RbpjKO;R26R mice with X-gal and the endocrine cell markers glucagon and insulin revealed that, in all three genetic backgrounds, some endocrine cells were not stained with X-gal, suggesting that these cells derived from epithelial cells that did not express, or only for a short time expressed, PTF1A (Fig. 5M-U, arrows). Conversely, we also found X-gal<sup>+</sup> islets in all lines.
suggesting that incomplete deletion is not selected for in any genetic background (data not shown). Importantly, we found no difference in morphology or cellular composition between X-gal-negative and X-gal-positive islets in the different genetic backgrounds.

DISCUSSION

The regulation of organogenesis and proper cell fate determination in the pancreas has been found to involve the activation of Notch signaling. To elucidate the role of epithelia expressed Notch receptors and the specific role of the transcription factor Rbpj as a transducer of Notch signaling, we used a conditional gene-targeting approach to genetically inactivate either Notch1 and Notch2 or Rbpj in the pancreas.

Recently, two different mouse models for conditional genetic inactivation of Rbpj were described, using either transgenic Pdx1-Cre or Ptf1a-Cre knockin mice for the targeting of pancreatic progenitor cells (Fujikura et al., 2006; Fujikura et al., 2007). During early pancreatic development, both models, and our RbpjKO mice, revealed an essential role for Rbpj with premature glucagon+ cell development, a severe decrease in acinar cell differentiation and disturbed ductal branching in mutant mice. Differences in the phenotypical severity between these models during the early stages of organogenesis are possibly due to differences in the onset, timing and rate of Cre-induced genetic inactivation. In our Ptf1a+/Creex1 mice, X-gal staining in the buds was observable at E10.5 in all pancreatic epithelial cells, similar
to that previously reported (Fujikura et al., 2007; Kawaguchi et al., 2002). However, even slight differences in targetting efficiency during early pancreatic organogenesis in transgenic Pdx1-Cre mice, and between different Ptf1a-knock-in Cre lines, may have a large impact on the development of the respective cell compartments.

Of interest is the appearance of late exocrine cells in all models; however, phenotypical effects are notable. In our model, RbpjKO mice do not survive more than 4-5 days after birth, most probably as a result of the clinically apparent pancreatic insufficiency with impaired weight gain, a high content of milk in the stomach of animals and no apparent neurological phenotype. We favor this explanation over, for example, extra-pancreatic causes, as we could not detect any defects in other PTF1A-expressing organs, such as the retina or the CNS (data not shown). The reason why our RbpjKO mice are not able to show the same ability to develop an apparently normal adult exocrine compartment is not clear, but may possibly be explained by a more rigorous deletion of early progenitors in our mice. Nevertheless, the late appearance of acinar cells during organogenesis in our and other Rbpj-deficient pancreata (Fujikura et al., 2006; Fujikura et al., 2007) is surprising, and may occur through Rbpj-independent mechanisms involving a recently identified regulator of acinar cell development, the Rbpj homolog Rbpj1 (Beres et al., 2006). These authors showed that the initiation of the acinar differentiation program by the PTF1 complex involves RBPJ binding to PTF1A to form the PTF1-J complex. This complex then activates RBPJL, which itself binds to PTF1A to form the PTF1-L complex. PTF1-L has been shown to be the more active complex, activating acinar genes such as amylase and elastase (Beres et al., 2006). The finding of delayed expression of acinar genes such as amylase at E18.5 in RbpjKO mice may be explained by two mechanisms. First, Cre activation may not be complete in a few proacinar cells, which will eventually form the exocrine organs. However, our results showing Cre-induced recombination of both Rbpj alleles in microdissected acini and in adult pancreatic tissue (Fujikura et al., 2007) do not support this hypothesis. Secondly, spontaneous activation of Rbpj1 in precursor cells expressing PTF1A may lead to the formation of PTF1-L and, thus, to a positive-feedback loop activating the Rbpj1 promoter. The delayed appearance and the small initial population of acinar cells would be consistent with a stochastic activation of Rbpj1, a hypothesis as yet unproven however.

The defective ductal branching observed in our, as well as in other, models of Notch signaling ablation may be due to early reduction of the epithelial progenitor pool, as has been suggested previously (Fujikura et al., 2006; Fujikura et al., 2007). Interestingly, the ductal cells in RbpjKO and Notch1/2KO mice expressed CK19, suggesting that the differentiation of progenitor cells into ductal cells is not inhibited by inactivated Rbpj-dependent Notch signaling. Future studies may help to determine the factors regulating ductal differentiation in the pancreas.

Of unclear significance is our finding of amylase positivity in ductal structures of RbpjKO pancreata. Although we detected amylase" cells within the ducts, we did not detect expression of PTF1A in these cells (data not shown); however, expression of these acinar transcription factors may be below detection limits. Other explanations include artificial staining of amylase produced by extraductal acinar cells; however, we did not observe acini in the dorsal part of the organ. Whether or not inactivated Notch signaling contributes to acinar cell fate determination from ductal cells or within ductal structures needs to be determined.

Interestingly, we found differences between Notch1/Notch2 and Rbpj ablated mice regarding the severity of impaired pancreatic development. Whereas the buds of Notch1/2KO mice appeared smaller than those of wild-type littermates, this reduction did not reach the extent of that seen in RbpjKO mice. The reason for the reduced proliferation of pancreatic progenitors may be due to the requirement of Notch signals for the maintenance of actively proliferating pancreatic progenitor cells, as has recently been shown for the transcription factor Sox9 (Seymour et al., 2007). In this regard, we also found reduced, but not completely abolished, expression of HES1 in Notch1/2KO and RbpjKO mice, as has been noted by others (Fujikura et al., 2006; Fujikura et al., 2007), possibly as a result of the expression of factors such as Sox9, which is necessary for the maintenance of HES1 expression. Studies with ectopic overexpression of Notch1 showed the prevention of exocrine and endocrine differentiation of pancreatic progenitor cells, leaving them in an undifferentiated state (Esni et al., 2004; Hald et al., 2003; Murga et al., 2003). Despite technical issues, such as the transgenic expression and potentially non-physiological Notch1 levels, these results, as well as the aforementioned studies, point to a role for Notch in the regulation of pancreatic progenitor cells, with one of the main conclusions being a premature endocrine switch caused by insufficient Notch signaling. Interestingly however, we found such a switch in RbpjKO but not Notch1/2KO mice, possibly indicating the requirement of Rbpj but not Notch1 or Notch2 for the regulation of premature endocrine differentiation. However, we cannot rule out inefficient early Cre-induced inactivation of Notch1 and Notch2. While we could determine successful recombination of both Notch1 and Notch2 alleles at E12.5 by PCR of microdissected epithelial cell, incomplete earlier inactivation of both Notch genes might indeed be responsible for the lack of effect in Notch1/2KO mice. The modest phenotype of Notch1/2KO mice was unexpected and is in contrast to the skin, where genetic inactivation of Rbpj and Notch1/Notch2 leads to similar phenotypes (Schouwey et al., 2006). While early reports using mice null for Notch signaling family members, such as Rbpj, Dll1 or Hes1, showed impaired growth and branching defects (Apelqvist et al., 1999; Jensen et al., 2000), differences between null mice and conditional genetic targeting approaches, such as the inactivation of targeted genes before pancreatic development is started and the additional targeting of extra-pancreatic cells in null mice, has a strong impact on the observable phenotype.

The different impact of pancreatic Notch1/Notch2 and Rbpj inactivation in our study strongly suggests a Notch-independent role of Rbpj in pancreatic organogenesis. The near complete absence of acinar cells until late gestation suggests that RBPjK is required for the formation of the acinar lineage. Recent studies have shown that RBPjK is the binding partner of PTF1A for formation of the early PTF1-J complex (Beres et al., 2006; Masui et al., 2007; Obata et al., 2001). Our results are in line with a Notch-independent role of RBPjK as an obligate partner of PTF1A to form a functional PTF1 complex, a pivotal event during early pancreatic development. Thus, RBPjK in Notch1/2KO mice might still function as PTF1A-binding partner independently of its transducer role in the Notch signaling pathway.

As expected from previous reports (Apelqvist et al., 1999; Jensen et al., 2000), RbpjKO mice had earlier α cells underscoring the relevance of Notch signaling for the inhibition of premature differentiation of progenitor cells into early α cells. Our finding of less Ngn3" cells in RbpjKO mice is similar to the results by Fujikura and colleagues (Fujikura et al., 2006; Fujikura et al., 2007), and suggests that these endocrine progenitor cells are also
compromised and forced into premature differentiation by Rbpj deficiency. Notably, Notch1/2KO mice revealed no significant decrease in NGN3+ cells and several mechanisms may account for this finding. First, RBPJκ might be activated independently and might lead to the activation of target genes; second, the four Notch1 and Notch2 alleles might not be inactivated in a timely manner to preserve the progenitor pool; or third, the transition of the repressor into an activator state of RBPJκ may be, at least partially, Notch independent. Despite the premature differentiation of pancreatic progenitors in Rbpj-deficient mice, we found that all endocrine lineages develop in RbpjKO and Notch1/2KO mice, consistent with the hypothesis of a dispensable role of Notch signaling in late pancreatic development. However, we found fewer islets in both knockout lines, which, more prominently in RbpjKO mice, had a partially disturbed composition. One explanation for the development of endocrine cells despite the genetic inactivation of Notch signaling is the later expression of Cre recombinase in Ptf1a-Cre compared with in Pdx1-Cre mice at E10.5. However, development of the endocrine compartment and islet formation does not occur before E13.5, a time point at which Rbpj and both Notch genes are inactivated. Our finding of X-gal+ islets composed of all genes in Notch1/2KO mice are obtained from the Developmental Studies Hybridoma Bank, generous gift of PDX1 and HES1 antibodies, respectively. The CK19 antibody was provided by Jack Favor, Axel Walch and Andreas Voss for help with laser microcopy. We are grateful to John Fuxe, Michael Desoye, and Andreas Favor for help with laser capture microscopy. We also thank Raymond J. MacDonald and Saadettin Sel for helpful discussions, and Christopher V. Wright and Tetsuo Sudo for their generous gift of PDX1 and HES1 antibodies, respectively. The Cki.9 antibody developed by Rolf Kemler and the Neurogenin antibody developed by O. D. Madsen were obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa. This work was supported in part by grants from the Deutsche Forschungsgemeinschaft to R.M.S. (SFB 4456 and SFB576), U.Z.-S. and L.S. (STR-4613-2-2).

In conclusion, we demonstrate an essential role of Rbpj, but not of Notch1 and Notch2, in pancreatic organogenesis. Using a concomitant approach of Notch signaling inactivation, we show that the epithelially expressed Notch receptors 1 and 2 are not essential for pancreatic development, whereas lack of Rbpj leads to premature differentiation of pancreatic progenitors and a decrease in endocrine progenitor cells. During late pancreatic development, however, differentiated exocrine and endocrine lineages mature in both knockout lines. Although Rbpj seems to be an important regulator of the early pancreatic progenitor pool, our findings strengthen the hypothesis of as yet unknown and potentially Rbpj-independent mechanisms regulating the cell fate of adult pancreatic cell lineages. As we can show successful inactivation of Notch1 and Notch2 alleles at E12.5, this finding strongly suggests that these receptors, but not Rbpj, are dispensable for exocrine and endocrine development. Thus, at least in the pancreas, a Notch-independent role of Rbpj during development seems to be a likely mechanism.

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References