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Mind bomb 1 is required for pancreatic β-cell formation

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During early pancreatic development, Notch signaling represses differentiation of endocrine cells and promotes proliferation of Nkx6-1$^+$Ptf1a$^+$ multipotent progenitor cells (MPCs). Later, antagonistic interactions between Nkx6 transcription factors and Ptf1a function to segregate MPCs into distal Nkx6-1$^-$Ptf1a$^+$ acinar progenitors and proximal Nkx6-1$^+$Ptf1a$^-$ duct and β-cell progenitors. Distal cells are initially multipotent, but evolve into unipotent, acinar cell progenitors. Conversely, proximal cells are bipotent and give rise to duct cells and late-born endocrine cells, including the insulin producing β-cells. However, signals that regulate proximodistal (P-D) patterning and thus formation of β-cell progenitors are unknown. Here we show that *Mind bomb 1* (*Mib1*) is required for correct P-D patterning of the developing pancreas and β-cell formation. We found that endoderm-specific inactivation of *Mib1* caused a loss of Nkx6-1$^+$Ptf1a$^-$ and Hnf1β$^+$ cells and a corresponding loss of Neurog3$^+$ endocrine progenitors and β-cells. An accompanying increase in Nkx6-1$^-$Ptf1a$^+$ and amylase$^+$ cells, occupying the proximal domain, suggests that proximal cells adopt a distal fate in the absence of Mib1 activity. Impeding Notch-mediated transcriptional activation by conditional expression of dominant negative Mastermind-like 1 (Maml1) resulted in a similarly distorted P-D patterning and suppressed β-cell formation, as did conditional inactivation of the Notch target gene *Hes1*. Our results reveal iterative use of Notch in pancreatic development to ensure correct P-D patterning and adequate β-cell formation.

diabetes | lateral signaling | tip | trunk

The pancreas arises from two outgrowths on the foregut endoderm around embryonic day (E)9.0. The early pancreatic epithelium consists of multipotent progenitor cells (MPCs) that express the transcription factors Pdx1, Ptf1a, and Nkx6-1 (1). Neurog3-expressing cells, arising in the dorsal anlage around E9.0, mark the beginning of endocrine differentiation (1). Early pancreas development, known as the "primary transition," is characterized by the cells being in a "protodifferentiated state" with low level expression of genes encoding differentiation markers. The "secondary transition," characterized by high expression of differentiation markers and appearance of zymogen granules in the acinar cells, begins around E14.5 (2).

Mutation of the Notch pathway genes *Dll1*, *Rbpj*, and *Hes1* results in excessive endocrine development at the primary transition (3, 4). Ptf1a is required for expression of the Notch ligand Dll1 in MPCs, ensuring normal proliferation of MPCs independently of the repressive effect on endocrine differentiation (5). Conditional loss-of-function studies have been used to examine the role of Notch signaling in later pancreatic development. *Pdx1*-Cre mediated inactivation of *Rbpj* caused accelerated α-cell differentiation but this was followed by decreased numbers of Neurog3$^+$ cells at E11.5 (6). At E15, tubular structures expressing ductal markers were seen, whereas differentiation of acinar and all types of endocrine cells were reduced. However, Rbpj acts as a repressor in the absence of Notch signaling in addition to its role in Notch target gene activation (7, 8). Its removal, therefore, does not necessarily reflect the lack of Notch pathway activity, but rather a combination of derepression and loss-of-activation states. Also, Rbpj is a component of the Ptf1 complex (9, 10) making it difficult to establish whether defects in *Rbpj*-deficient embryos is caused by altered Notch signaling or by loss of Ptf1 function or both.

MPCs in the growing epithelium gradually segregate into discrete proximal and distal domains (1, 11, 12). Antagonistic interactions between Nkx6 transcription factors and Ptf1a resolve Nkx6-1$^+$Ptf1a$^+$ MPCs into distal Nkx6-1$^-$Ptf1a$^+$ acinar progenitors and proximal Nkx6-1$^+$Ptf1a$^-$ duct and β-cell progenitors (11). This proximodistal (P-D) patterning of the pancreatic epithelium appears to be complete around E13.5–E14.5 (11, 12), but little is known about the signals that control this process. Expression of constitutively active Notch1 intracellular domain (NICD) in embryonic pancreas blocks both endocrine and exocrine differentiation (13, 14) and favors an Nkx6-1$^+$Ptf1a$^-$ state (11). However, relatively normal pancreas development is observed after *Ptf1a*-Cre–mediated deletion of *Notch1/2* (15). A more recent study found that a certain level of presenilin activity is required in endocrine progenitors in order for these to retain their endocrine lineage choice. Presenilins (Ps1 and Ps2) are components of the γ-secretase complex that cleaves Notch receptors upon ligand-mediated activation (16), and in embryos with inactivation of three or more *presenilin1/2* alleles (Ps^{Lo} embryos) the endocrine progenitors, identified by *Neurog3*-Cre–mediated lineage tracing, adopt an acinar fate (17). A genetic interaction with Notch2 indicated that Notch was the relevant substrate in this process. Intriguingly, mouse embryos with lower than normal Neurog3 protein levels (Neurog3^{Lo} cells) also redirect a large number of *Neurog3*-Cre lineage traced cells to an acinar fate (18). Whether the redirection of *Neurog3*-Cre–expressing cells in Ps^{Lo} embryos is associated with increased numbers of Neurog3^{Lo} cells is unknown.

Mind bomb 1 (*Mib1*) encodes an E3 ubiquitin ligase essential for Notch ligand activity (19–21) and we show here that endodermal

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inactivation of *Mib1* causes a loss of Nkx6-1$^+$Ptf1a$^-$ cells and a corresponding loss of duct cells, Neurog3$^+$ endocrine progenitors, and β-cells. An accompanying increase in Nkx6-1$^-$Ptf1a$^+$, Carboxypeptidase A (Cpa)$^+$, and amylase$^+$ cells in the proximal domain suggests that proximal cells adopt a distal fate in the absence of Mib1 activity. Attenuation of Notch-mediated transcriptional activation by conditional expression of dominant negative Mastermind-like 1 (Maml1) in endoderm caused similarly distorted P-D patterning and suppressed β-cell formation as did endodermal inactivation of the Notch target gene *Hes1*. Our results demonstrate that Notch signaling is required to prompt MPCs to adopt a proximal fate and thereby for generation of adequate numbers of duct and β-cells.

Results

Notch Signaling Is Required for β-Cell Formation. To avoid early embryonic lethality (3, 4, 20, 22) and to circumvent redundancy between Notch ligands, we made *Foxa2*^{T2AiCre} mice by homologous recombination (Fig. S1) and crossed them with mice carrying floxed *Mib1* alleles (21) to inactivate *Mib1* in definitive endoderm (*Mib1*$^{\Delta Edd}$). Early β-galactosidase activity in the entire endoderm of *Foxa2*^{T2AiCre}; R26R (23) embryos demonstrates efficient recombination by the *Foxa2*^{T2AiCre} allele (Fig. S1). Pdx1$^+$ pancreatic endoderm was specified normally in E9.5 *Mib1*$^{\Delta Edd}$ embryos, showing that the inductive property of the notochord is not affected by loss of Mib1 activity. E9.5 *Mib1*$^{\Delta Edd}$ embryos had an excess of Neurog3$^+$ cells in the dorsal bud, as expected from loss of Notch signaling (3–6, 24), and a near complete conversion of the dorsal bud to glucagon$^+$ cells at E10.5 (Fig. S2). A dorsal pancreas could not be found in *Mib1*$^{\Delta Edd}$ embryos after E10.5, most likely as a result of the strong endocrinogenic phenotype. In contrast, the ventral anlage developed relatively normally (Fig. S2) and we thus examined the appearance of the ventral pancreas in later stage *Mib1*$^{\Delta Edd}$ embryos. To learn the status of Notch signaling, we examined Hes1 expression by immunofluorescent (IF) laser-scanning microscopy (LSM) analysis. E12.5 and E15.5 *Mib1*$^{\Delta Edd}$ embryos lost Hes1 expression in Pdx1$^+$ pancreatic endoderm (Fig. S3), showing that Notch signaling is impeded (5). Remarkably, we found a total loss of insulin producing cells in E18.5 *Mib1*$^{\Delta Edd}$ embryos as well as a loss of Dolichos biflorus agglutinin (DBA)$^+$ duct structures (Fig. 1 *A* and *B* and Fig. S4). We then examined *Mib1*$^{\Delta Edd}$ embryos at E15.5 where β-cell formation is peaking (1) and found a total absence of insulin$^+$ cells in *Mib1*$^{\Delta Edd}$ embryos compared with controls (Fig. 1 *E–G*). Quantitative real-time RT-PCR (qPCR) showed a strong reduction in *Ins1*, *Sst*, and *Ppy* expression (Fig. 1*H*), confirming the loss of β-cells and suggesting that other late-born endocrine cells are also reduced. In contrast, *Gcg* expression was not significantly changed.

Apart from its role in Notch signal-sending cells (19–21), Mib1 regulates cellular levels of the death-associated protein kinase (DAPK) (25), Ryk-dependent Wnt/β-catenin signaling (26), and the innate immune response to RNA vira (27). Among these activities, dysregulation of Notch and/or Wnt/β-catenin signaling is most likely to affect pancreatic development. However, loss of Wnt/β-catenin signaling in the developing pancreas results in a paucity of acinar development, the opposite of what we observe in *Mib1*$^{\Delta Edd}$ embryos (28), arguing against involvement of this pathway. Nevertheless, to more firmly establish whether the β-cell loss observed in conditional *Mib1*$^{\Delta Edd}$ mutants was due to deficient Notch signaling we quantified β-cell development in embryos where the transcriptional activity of Notch was blocked in the endoderm by *Foxa2*^{T2AiCre}-induced expression of a dominant negative Maml1-EGFP fusion protein from a targeted *Rosa26* locus (*R26*^{dnMaml1}; Fig. S5). This fusion protein is a potent and specific inhibitor of all four mammalian Notch receptors in vivo (29–33), and as expected *R26*^{dnMaml1} embryos showed increased numbers of Neurog3$^+$ cells at E9.5 and increased glucagon$^+$ cells at E10.5 (Fig. S2). E12.5 and E15.5 *R26*^{dnMaml1} embryos showed uniform EGFP expression and strongly reduced Hes1 immunoreactivity in the pancreatic epithelium (Fig. S3). As shown in Fig. 1 *I–K*, the insulin$^+$ area was decreased by ~65% in E15.5 *R26*^{dnMaml1} embryos compared with controls, demonstrating that inhibition of Notch transcriptional activity reduces β-cell formation. qPCR analyses revealed a reduction in *Ins1*, *Sst*, and *Ppy* expression in *R26*^{dnMaml1} embryos compared with controls. In contrast to *Mib1*$^{\Delta Edd}$ embryos, *Gcg* expression was significantly reduced in *R26*^{dnMaml1} embryos (Fig. 1*L*).

To begin to determine the molecular mechanism by which Notch promotes β-cell formation, we next tested whether *Hes1*, a Notch target gene in the embryonic pancreas (4, 5), is required for efficient β-cell formation during the secondary transition. We used *Foxa2*^{T2AiCre} and *Hes1* floxed mice (34) to generate embryos where *Hes1* was inactivated in the definitive endoderm (*Hes1*$^{\Delta Edd}$). As expected, *Hes1*$^{\Delta Edd}$ embryos had increased numbers of Neurog3$^+$ cells at E9.5 and increased glucagon$^+$ cells at E10.5 (Fig. S2) and Hes1 immunoreactivity was eliminated in pancreatic epithelium of *Hes1*$^{\Delta Edd}$ embryos at E12.5 and E15.5 (Fig. S3). Similar to *R26*^{dnMaml1} embryos, IF LSM analysis of E15.5 *Hes1*$^{\Delta Edd}$ embryos revealed a ~65% reduction in insulin immunoreactive cells compared with controls (Fig. 1 *M–O*). qPCR showed a reduction in *Ins1* and *Sst* expression in *Hes1*$^{\Delta Edd}$ embryos compared with controls, whereas *Gcg* expression was unchanged (Fig. 1*P*). Consistent with the less severe phenotypes of *R26*^{dnMaml1} and *Hes1*$^{\Delta Edd}$ embryos at E15.5, we did observe insulin$^+$ and DBA$^+$ cells in E18.5 *R26*^{dnMaml1} and *Hes1*$^{\Delta Edd}$ embryos but fewer than in controls (Fig. S4). These results demonstrate that Notch signaling, acting through Hes1, is required for development of normal duct and β-cell numbers during the secondary transition.

Loss of Neurog3$^+$ Progenitors in *Mib1*$^{\Delta Edd}$, *R26*^{dnMaml1}, and *Hes1*$^{\Delta Edd}$ Embryonic Pancreas. To establish the cause of the β-cell loss in *Mib1*$^{\Delta Edd}$, *R26*^{dnMaml1}, and *Hes1*$^{\Delta Edd}$ embryos, we first quantified the number of Neurog3-expressing endocrine precursors by IF LSM analysis. The number of Neurog3$^+$ cells relative to total epithelial area was reduced by >99% in *Mib1*$^{\Delta Edd}$ embryos compared with controls at E15.5, and qPCR showed a severe reduction of *Neurog3* expression in E15.5 *Mib1*$^{\Delta Edd}$ embryos (Fig. 2 *A–D*), demonstrating that β-cell development in *Mib1*$^{\Delta Edd}$ embryos is arrested already at the precursor cell stage. Notably, the number of Neurog3$^+$ cells was reduced by >80% in E15.5 *R26*^{dnMaml1} and *Hes1*$^{\Delta Edd}$ embryos compared with controls and qPCR showed a reduction of *Neurog3* expression in E15.5 *R26*^{dnMaml1} and *Hes1*$^{\Delta Edd}$ embryos (Fig. 2 *E–L*). Together, these data demonstrate that the β-cell loss observed in conditional Notch pathway mutants can be explained by a failure to develop Neurog3$^+$ β-cell precursors. Loss of Neurog3$^+$ cells is also seen when apicobasal polarity is disturbed (35), but IF staining for the apical marker Muc1 did not uncover evidence of defective polarity in the pancreatic epithelium of *Mib1*$^{\Delta Edd}$, *R26*^{dnMaml1}, and *Hes1*$^{\Delta Edd}$ embryos (Fig. 2 *B*, *F*, and *J*).

Altered P-D Patterning in *Mib1*$^{\Delta Edd}$, *R26*^{dnMaml1}, and *Hes1*$^{\Delta Edd}$ Embryonic Pancreas. Constitutive Notch activity prevents acinar development and favors Nkx6-1 expression in pancreas epithelium (11, 13, 14). We thus examined whether the proximal, Nkx6-1$^+$Ptf1a$^-$ trunk epithelium, from which Neurog3$^+$ β-cell precursors arise (11, 12, 36), was properly established in *Mib1*$^{\Delta Edd}$ embryos. Double IF LSM detection of Nkx6-1 and Ptf1a expression revealed that Nkx6-1$^+$Ptf1a$^-$ cells were absent from the proximal domain of E15.5 *Mib1*$^{\Delta Edd}$ pancreas compared with controls (Fig. 3 *A–C*). The loss of Nkx6-1$^+$Ptf1a$^-$ cells was accompanied by a matching increase of Nkx6-1$^-$Ptf1a$^+$ cells in the proximal domain (Fig. 3 *A–C*). Moreover, Hnf1β$^+$ and DBA$^+$ cells were also lost in E15.5 *Mib1*$^{\Delta Edd}$ embryos and qPCR demonstrated a reduction in the expression of proximal markers *Nkx6-1*, *Hnf1b*, and *Sox9* (Fig. S6). Concurrently, Cpa$^+$ and amylase$^+$

DEVELOPMENTAL BIOLOGY

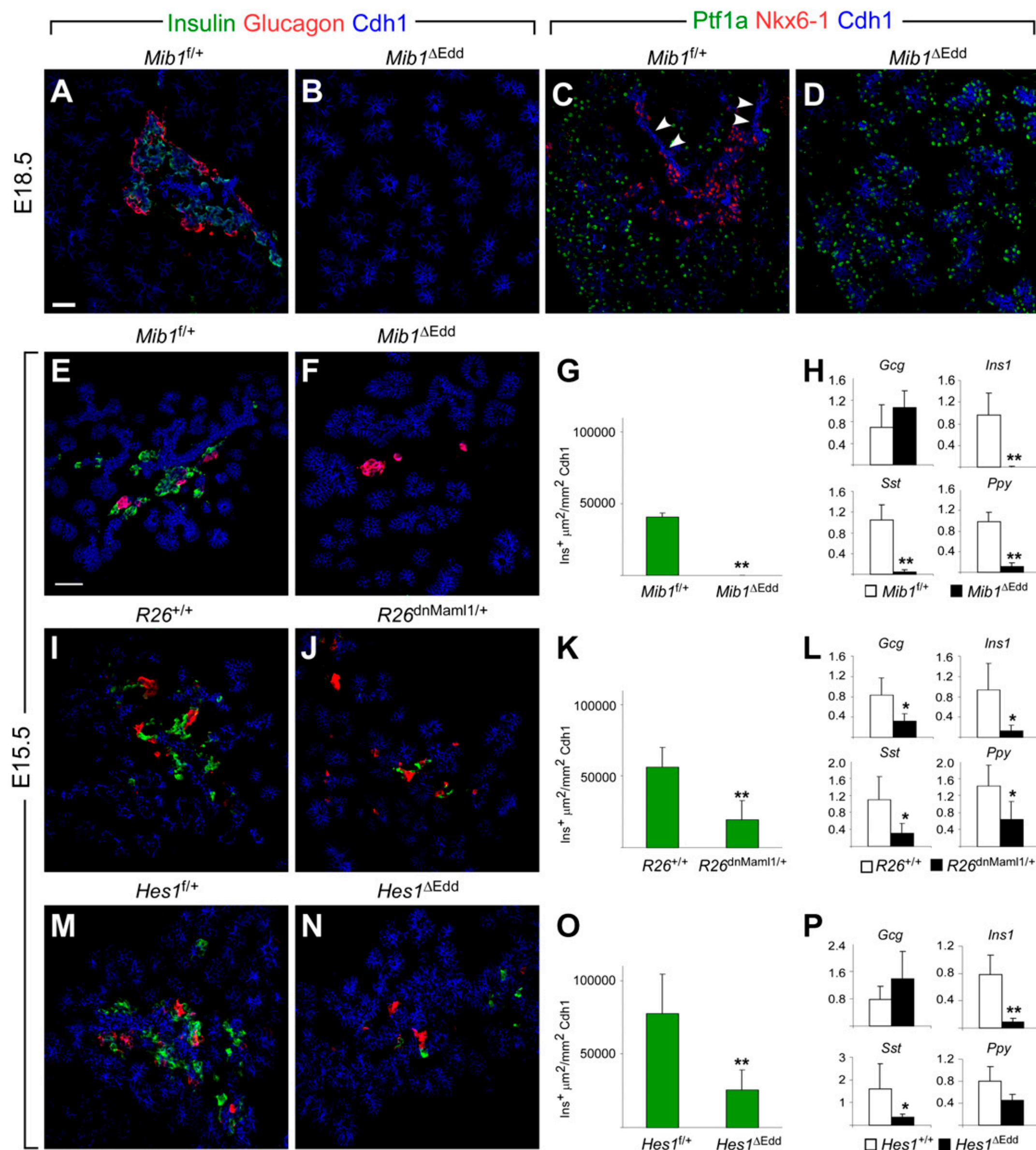

Fig. 1. β-Cell formation requires active Notch signaling. (*A* and *B*) Optical sections of E18.5 wild-type (*A*) and $Mib1^{\Delta Edd}$ (*B*) pancreata stained for insulin, glucagon, and Cdh1. Note complete loss of insulin$^+$ cells in $Mib1^{\Delta Edd}$ embryos. (*C* and *D*) Optical sections of wild-type (*C*) and $Mib1^{\Delta Edd}$ (*D*) embryos stained for Nkx6-1, Ptf1a, and Cdh1 as indicated. Note loss of Cdh1$^+$Nkx6-1$^-$Ptf1a$^-$ duct cells (arrowheads in *C*) in $Mib1^{\Delta Edd}$ embryos. (*E*, *F*, *I*, *J*, *M*, and *N*) Optical sections of E15.5 pancreata from $Mib1^{\Delta Edd}$ (*F*), $R26^{dnMaml1}$ (*J*), and $Hes1^{\Delta Edd}$ (*N*) embryos and controls (*E*, *I*, and *M*) stained for insulin, glucagon, and Cdh1 as indicated. (Scale bars, 50 μm.) (*G*, *K*, and *O*) Insulin$^+$ area in micrometers squared is shown relative to total pancreatic epithelial area in millimeters squared. (*H*, *L*, and *P*) qPCR analyses of *Glucagon* (*Gcg*), *Insulin1* (*Ins1*), *Somatostatin* (*Sst*), and *Pancreatic Polypeptide* (*Ppy*) expression at E15.5 in $Mib1^{\Delta Edd}$ (*H*), $R26^{dnMaml1}$ (*L*), and $Hes1^{\Delta Edd}$ (*P*) pancreata compared with controls. Data are presented as mean + SD. $^*P < 0.05$, $^{**}P < 0.01$.

cells were found in the proximal domain of E15.5 $Mib1^{\Delta Edd}$ embryos and qPCR showed increased expression of distal markers *Ptf1a*, *Mist1*, and *Cpa1* in E15.5 $Mib1^{\Delta Edd}$ embryos compared with controls (Fig. S7). Together these findings suggest that proximal cells adopt a distal cell fate in the absence of Mib1 activity.

Correspondingly, we found that the number of Nkx6-1$^+$Ptf1a$^-$ cells in E15.5 $R26^{dnMaml1}$ and $Hes1^{\Delta Edd}$ embryos was reduced by ~75 and ~85%, respectively, compared with controls (Fig. 3 *D*–*I*). An equivalent increase of Nkx6-1$^-$Ptf1a$^+$ cells occupying the proximal domain was also found in $R26^{dnMaml1}$ and $Hes1^{\Delta Edd}$ embryos (Fig. 3 *D*–*I*). Strikingly, E15.5 $Hes1^{\Delta Edd}$ embryos alone showed a significant presence of Nkx6-1$^+$Ptf1a$^+$ cells (Fig. 3*I*). As in $Mib1^{\Delta Edd}$ embryos, we found fewer Hnf1β$^+$ and DBA$^+$ cells (Fig. S6), whereas Cpa$^+$ and amylase$^+$ cells were found in the proximal domain of E15.5 $R26^{dnMaml1}$ and $Hes1^{\Delta Edd}$ embryos (Fig. S7). Furthermore, qPCR analyses revealed reduced expression of the proximal markers *Nkx6-1*, *Hnf1b*, and *Sox9* in $R26^{dnMaml1}$ and $Hes1^{\Delta Edd}$ embryos (Fig. S6), whereas expression of the distal markers *Ptf1a* and *Cpa1* were increased in $R26^{dnMaml1}$ embryos (Fig. S7). However, expression of distal markers was not increased in $Hes1^{\Delta Edd}$ embryos. Together these results demonstrate that Notch signaling regulates P-D patterning of the developing pancreas epithelium and suggest, together with the residual β-cell formation in $Hes1^{\Delta Edd}$ embryos, that Notch acts through Hes1-dependent as well as Hes1-independent mechanisms.

We next examined $Mib1^{\Delta Edd}$, $R26^{dnMaml1}$, and $Hes1^{\Delta Edd}$ embryos at E12.5, where Nkx6-1$^+$Ptf1a$^+$ MPCs have begun to resolve into single positive cells (11). Double IF LSM analyses

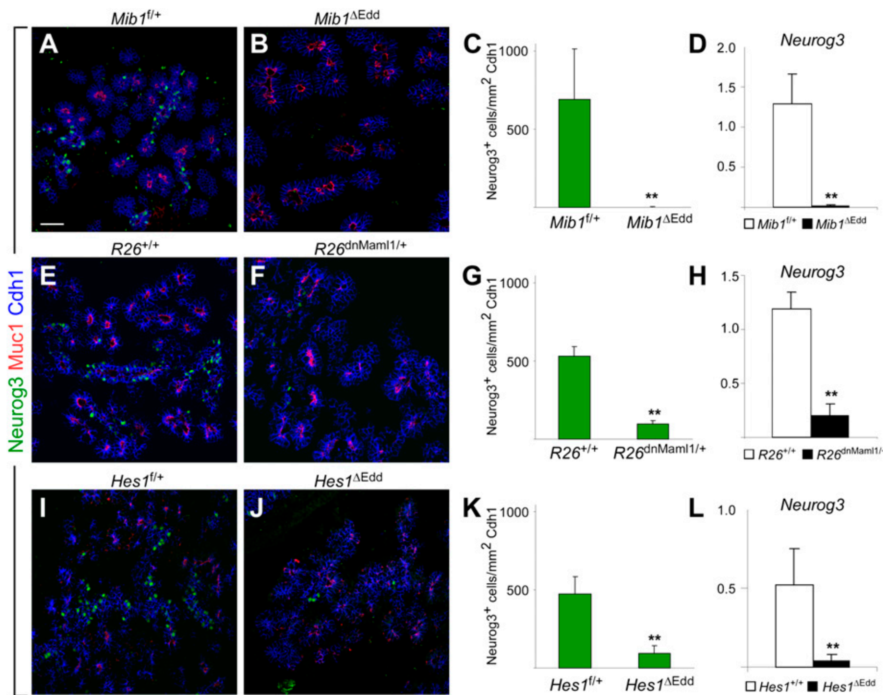

Fig. 2. Neurog3-expressing precursor cells are lost in $Mib1^{\Delta Edd}$ embryos. (*A*, *B*, *E*, *F*, *I*, and *J*) confocal optical sections of E15.5 pancreata from $Mib1^{\Delta Edd}$ (*B*), $R26^{dnMaml1}$ (*F*), and $Hes1^{\Delta Edd}$ (*J*) embryos and controls (*A*, *E*, and *I*) stained for Neurog3, Muc1, and Cdh1 as indicated. (Scale bar, 50 μm.) (*C*, *G*, and *K*) Number of $Neurog3^+$ cells is presented relative to total pancreatic epithelial area in mm^2. (*D*, *H*, and *L*) qPCR data for *Neurogenin 3* (*Neurog3*) expression at e15.5 in $Mib1^{\Delta Edd}$ (*D*), $R26^{dnMaml1}$ (*H*), and $Hes1^{\Delta Edd}$ (*L*) pancreata compared with controls. Data are presented as mean + SD. $*P < 0.05$, $**P < 0.01$.

showed that $Mib1^{\Delta Edd}$ embryos had the same number of Nkx6-$1^+$$Ptf1a^+$ cells as controls at E12.5 (Fig. 4 *A*–*C*). In contrast to E15.5 $Mib1^{\Delta Edd}$ embryos, Nkx6-$1^+$$Ptf1a^-$ cells were present in E12.5 $Mib1^{\Delta Edd}$ embryos but were reduced by >50% and we also noted a roughly corresponding increase of Nkx6-$1^-$$Ptf1a^+$ cells (Fig. 4 *A*–*C*). Similar changes were observed in E12.5 $R26^{dnMaml1}$ and $Hes1^{\Delta Edd}$ embryos, except that $Hes1^{\Delta Edd}$ embryos did not display increased numbers of Nkx6-$1^-$$Ptf1a^+$ cells (Fig. 4 *D*–*I*), possibly due to an earlier transient requirement for Hes1 to sustain Ptf1a expression in MPCs (5).

These results suggest that P-D patterning begin during the primary transition consistent with the rare occurrence of Nkx6-$1^-$$Ptf1a^+$ and Nkx6-$1^+$$Ptf1a^-$ cells in the E10.5 dorsal bud which otherwise contains mostly Nkx6-$1^+$$Ptf1a^+$ cells (11). Accordingly, we made embryos where *Dll1*, the only Notch ligand-encoding gene expressed in the primary transition pancreas (3, 37, 38) was deleted in the definitive endoderm ($Dll1^{\Delta Edd}$; Fig. S8) and examined P-D patterning and β-cell development in these embryos. E10.5 $Dll1^{\Delta Edd}$ embryos had excessive formation of $glucagon^+$ cells (Fig. S8), similar to what is observed in *Dll1* null embryos (5),

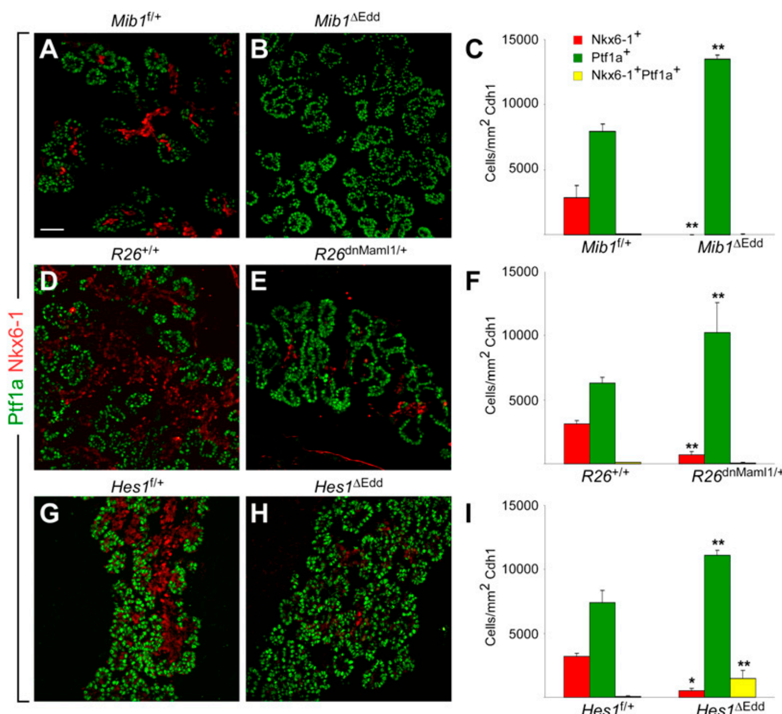

Fig. 3. Nkx6-$1^+$$Ptf1a^-$ precursor cells are lost from the central pancreatic epithelium. (*A*, *B*, *D*, *E*, *G*, and *H*) Confocal optical sections of E15.5 pancreata from $Mib1^{\Delta Edd}$ (*B*), $R26^{dnMaml1}$ (*E*), and $Hes1^{\Delta Edd}$ (*H*) embryos and controls (*A*, *D*, and *G*) stained for Nkx6-1 and Ptf1a as indicated. (Scale bar, 50 μm.) (*C*, *F*, *I*) Number of Nkx6-1^+, $Ptf1a^+$, and Nkx6-$1^+$$Ptf1a^+$cells is presented relative to total pancreatic epithelial area in millimeters squared. Data are presented as mean + SD. $*P < 0.05$, $**P < 0.01$.

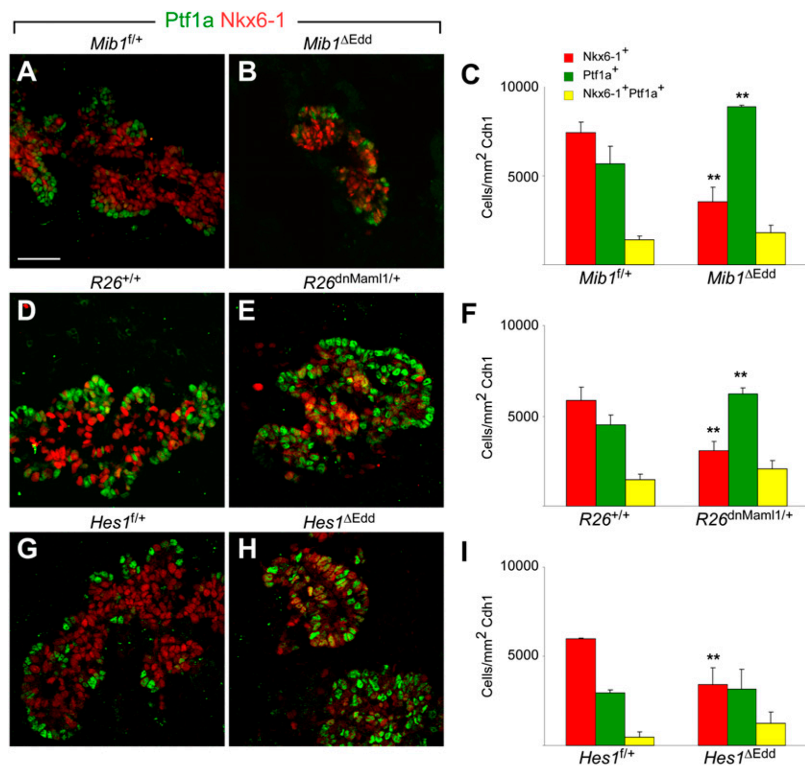

Fig. 4. Nkx6-1$^+$Ptf1a$^-$ precursor cells are reduced at E12.5. (*A*, *B*, *D*, *E*, *G*, and *H*) Confocal optical sections of E12.5 pancreata from $Mib1^{\Delta Edd}$ (*B*), $R26^{dnMaml1}$ (*E*), and $Hes1^{\Delta Edd}$ (*H*) embryos and controls (*A*, *D*, and *G*) stained for Nkx6-1 and Ptf1a as indicated. (Scale bar, 50 μm.) (*C*, *F*, and *I*) Number of Nkx6-1$^+$, Ptf1a$^+$, and Nkx6-1$^+$Ptf1a$^+$cells is presented relative to total pancreatic epithelial area in millimeters squared. Data are presented as mean + SD. $*P < 0.05$, $**P < 0.01$.

indicating that recombination of the floxed *Dll1* allele was efficient. We saw reduced Nkx6-1$^+$Ptf1a$^-$ cell numbers in E12.5 $Dll1^{\Delta Edd}$ embryos, whereas the number of Nkx6-1$^-$Ptf1a$^+$ cells did not change significantly (Fig. S8), suggesting that Dll1 may be involved in the initiation of P-D patterning in the early pancreatic epithelium. However, we found no apparent defects in P-D patterning in E15.5 and E18.5 $Dll1^{\Delta Edd}$ embryos (Fig. S8), suggesting that other Notch ligands expressed in the secondary transition pancreas (38, 39) can provide the necessary ligand activity. Consistent with this notion we previously found that loss of NICD immunoreactivity and *Hes1* expression observed in E10.5 Dll1$^{-/-}$ embryos recovers at E11.5 (5). Furthermore, these results show that an early endocrinogenic phenotype caused by loss of Dll1 is not causing a later P-D patterning defect.

Discussion

Here we show that the MPCs of the early pancreatic epithelium fail to segregate into discrete proximal and distal fates when Notch activity is attenuated. Instead, most of the epithelium adopts a distal fate and the resulting loss of Nkx6-1$^+$Ptf1a$^-$ cells thwarts development of Neurog3$^+$ endocrine progenitors and β-cells. Together with other recent studies (5, 40), our results suggest that Notch is used iteratively to control cell fate choices during pancreatic development (Fig. S9). Our conditional mutants displayed the expected phenotype with excess Neurog3$^+$ cells at E9.0–E9.5 and excess glucagon$^+$ cells at E10.5 and this enhanced formation of glucagon$^+$ cells likely explains the presence of glucagon$^+$ cells at late embryonic stages.

Our data are consistent with the reduction of endocrine cells seen in pancreas-specific *Rbpj* mutants (6) and suggest that allocation of MPCs to a proximal fate is perturbed in these embryos. Conversely, mutations resulting in gain of Notch signaling, such as forced expression of NICD in the pancreatic epithelium (11), display an opposite phenotype with loss of Ptf1a expression. Equally, embryos with a mutation of *Sel1l*, which encodes an inhibitor of Notch signaling, show a remarkably selective loss of Ptf1a$^+$ and amylase$^+$ cells (41).

Ptf1a-Cre–mediated deletion of Notch1/2 has little influence on late pancreatic development including β-cell formation (15), apparently contradicting our results. However, efficient recombination of Notch1/2 alleles was only observed at E12.5, after MPCs begin to resolve into distinct Nkx6-1$^+$ and Ptf1a$^+$ progenitors and may therefore preferentially affect distal progenitors. This late deletion combined with the choice of deleting Notch1/2, which will only affect signal receiving cells, may have masked the role of Notch in late pancreatic development. Indeed, the proximal-to-distal direction of the fate change seen in the present study suggest that Ptf1a$^+$ progenitors are signal sending rather than signal receiving, consistent with Ptf1a being an important activator of *Dll1* expression in MPCs (5).

Our results are similar to those of Cras-Meneur et al. who observed a conversion of Neurog3-Cre lineage traced endocrine progenitors into acinar cells when deleting three or more alleles of *Presenilin1/2* (17). This phenotype was interpreted as a transient requirement for Notch signaling in committed Neurog3$^+$ endocrine progenitors to maintain the endocrine fate. However, mouse embryos with lower than normal Neurog3 protein levels (Neurog3^{Lo} cells) also redirect a large number of *Neurog3*-Cre–expressing cells to an acinar fate (18). It is thus possible that the redirection of *Neurog3*-Cre expressing cells in Ps^{Lo} embryos can be explained by increased formation of Neurog3^{Lo} cells, as expected from a classical Notch pathway mutation. In this regard, it is noteworthy that Ps^{Lo} embryos displayed a 4.5-fold increase of Neurog3$^+$ cells at E18.5 (17). Unfortunately, earlier stages were not analyzed for Neurog3 expression.

The $Foxa2^{T2AiCre}$ line is also active in heart and notochord and the phenotypes observed could be due to loss of Notch signaling there, as both of these tissues are involved in inductive interactions with foregut endoderm (42, 43). However, the specification of the pancreas (and liver) endoderm occurs normally in our conditional mutants, indicating that the inductive properties of these tissues are intact and that the defects observed in pancreatic development is a result of a cell autonomous requirement for Notch signaling in the pancreatic endoderm. Other

endodermal tissues such as stomach and duodenum also showed defective development but a detailed description of these phenotypes is beyond the scope of this paper.

We anticipate that our findings will advance efforts to generate β-cells from human ES cells. Many protocols designed to induce development of insulin producing β-cells from human ES cells attempt to induce β-cell formation by inhibiting Notch activity with γ-secretase inhibitors (44–47). Such protocols should now be reconsidered in light of our findings. We propose that maintenance of active Notch signaling in human ES cell-derived pancreatic progenitors, until they reach an $Nkx6\text{-}1^{+}Ptf1a^{-}$ stage, will be beneficial for the development of mature β-cells.

Methods

Mice. *Mib1*$^{f/f}$ and *Hes1*$^{f/f}$ were maintained as previously described (21, 34). Genotyping of *Dll1*$^{f/f}$, *R26*^{dnMaml1}, and *Foxa2*^{T2AiCre} was done using PCR (primers sequences are available upon request). *Dll1*$^{f/f}$, *Mib1*$^{f/f}$, and *Hes1*$^{f/f}$ strains were maintained as homozygous, whereas *R26*^{dnMaml1} and *Foxa2*^{T2AiCre} were kept as heterozygous. Generation of targeting constructs and homologous recombination is described in *SI Methods*. Animals were maintained in adherence to guidelines issued by the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123).

Analytical Methods. Histological analysis including antibodies used, western blotting, and quantitative real-time RT-PCR methods are described in detail in *SI Methods*.

Statistical Analysis. Statistical analysis was performed using a two-tailed Student's *T* test for equal variances. An *F* test was used to ascertain equal variances.

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