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On p. 36, Fig. 1 was incorrectly cited several times in place of Fig. 2. The correct paragraph appears below.

The authors apologise to readers for this mistake.

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Ptf1a-mediated control of Dll1 reveals an alternative to the lateral inhibition mechanism

Jonas Ahnfelt-Rønne1,*, Mette C. Jørgensen1,*, Rasmus Klinck1, Jan N. Jensen1, Ernst-Martin Füchtbauer2, Tye Deering3, Raymond J. MacDonald3, Chris V. E. Wright4, Ole D. Madsen1 and Palle Serup1,‡

SUMMARY
Neurog3-induced Dll1 expression in pancreatic endocrine progenitors ostensibly activates Hes1 expression via Notch and thereby represses Neurog3 and endocrine differentiation in neighboring cells by lateral inhibition. Here we show in mouse that Dll1 and Hes1 expression deviate during regionalization of early endoderm, and later during early pancreas morphogenesis. At that time, Ptf1a activates Dll1 in multipotent pancreatic progenitor cells (MPCs), and Hes1 expression becomes Dll1 dependent over a brief time window. Moreover, Dll1, Hes1 and Dll1/Hes1 mutant phenotypes diverge during organ regionalization, become congruent at early bud stages, and then diverge again at late bud stages. Persistent pancreatic hypoplasia in Dll1 mutants after eliminating Neurog3 expression and endocrine development, together with reduced proliferation of MPCs in both Dll1 and Hes1 mutants, reveals that the hypoplasia is caused by a growth defect rather than by progenitor depletion. Unexpectedly, we find that Hes1 is required to sustain Ptf1a expression, and in turn Dll1 expression in early MPCs. Our results show that Ptf1a-induced Dll1 expression stimulates MPC proliferation and pancreatic growth by maintaining Hes1 expression and Ptf1a protein levels.

KEY WORDS: Dll1, Hes1, Neurog3, Notch, Pancreas, Ptf1a, Mouse

INTRODUCTION
Onset of Pdx1 expression in mouse posterior foregut endoderm around E8.0 marks the initiation of pancreas development. The first Neurog3+ endocrine precursors (Gu et al., 2002) are found slightly later, between embryonic day (E) 8.5 and E8.75 (Jorgensen et al., 2007). Neurog3 is required and sufficient for induction of pancreatic endocrine development (Apelqvist et al., 1999; Gradwohl et al., 2000; Schwitzgebel et al., 2000). Early Neurog3+ precursors are increased in embryos deficient for the Notch pathway genes Dll1, Rbpj and Hes1 (Apelqvist et al., 1999; Jensen et al., 2000b), and pancreatic expression of the Notch1 intracellular domain (NICD) represses endocrine as well as exocrine differentiation (Esi et al., 2004; Hald et al., 2003; Mutraugh et al., 2003). These findings led to the suggestion that Notch-mediated lateral inhibition prevents excessive endocrine differentiation of progenitor cells, thereby allowing ensuing proliferation and morphogenesis of pancreatic progenitor cells (Apelqvist et al., 1999; Edlund, 2002; Jensen et al., 2000b; Skipper and Lewis, 2000).

The lateral inhibition model posits that onset of Neurog3 expression in multipotent pancreatic progenitor cells (MPCs) initiates endocrine differentiation and activates expression of the Notch receptor ligand Dll1. Dll1 subsequently activates Notch receptors in neighboring cells, turning on Hes1 expression in these. Hes1 then inhibits Neurog3 expression, preventing adjacent cells from adopting an endocrine fate. However, the lateral inhibition model is largely based on the similar phenotypes seen in the above-mentioned mutants and most of the model’s mechanistic predictions have not been tested rigorously. Also, major gaps exist in our understanding of the timing and extent of ligand expression and how it affects the behavior and number of MPCs as well as their progression to the defined endocrine/duct progenitors in the central epithelium versus acinar/duct progenitors of the peripheral epithelium at later stages (Kopinke et al., 2011; Schaffer et al., 2010). Further, recent studies imply that Notch-mediated regulation of endocrine differentiation is more complex than the model suggests. For example, while endocrine cells are formed continuously from E9.5 until birth, with a major burst in β-cell generation between E13.5 and E16.5 in the mouse, Notch ligand-receptor interactions are thought to change as development proceeds, either by temporally controlled activation of different ligands or through different affinities for different Notch ligand-receptor pairs.

Dll1 is the first ligand to be expressed in the pancreatic epithelium, together with the receptors Notch1 and Notch2, and Dll1 appears to be the only ligand expressed between E9.5 and E11.5 (Apelqvist et al., 1999; Lammert et al., 2000). However, Jag1 expression begins around E12 and becomes the most abundant ligand in the pancreas epithelium at mid-gestation (Golson et al., 2009; Lammert et al., 2000). Also, while a Ptf1aCre-mediated, pancreas-specific knock-out of Rbpj (Fujikura et al., 2006) shows increased numbers of Neurog3+ cells at E10.5, these animals showed decreased Neurog3+ cells at E11.5, and the pancreas was not hypoplastic as seen in Hes1 mutants (Jensen et al., 2000b). It was suggested that progenitors competent to initiate endocrine development become depleted very early and quite specifically if Notch signal transduction is compromised. Surprisingly, a recent study showed that a pancreas-specific compound knock-out of Notch1/2 had a very mild pancreatic phenotype without signs of deregulated Neurog3 expression (Nakahai et al., 2008). However, this mild phenotype may be due to...
timing of deletion, which was only shown to be efficient by E14.5, and/or due to possible compensatory activation of Notch3/4 expression in the pancreatic epithelium, which was not excluded. The function of Hes1 downstream of Dll1-Notch-mediated lateral inhibition may not be the only function of Hes1 in pancreatic development. For example, Hes1 mutants show ectopic pancreas formation (Fukuda et al., 2006; Sumazaki et al., 2004), but this has not been observed in other Notch pathway mutants. Furthermore, even though Hes1 mRNA expression appeared downregulated in the dorsal pancreas endoderm in Dll1 mutants at E9.5 (Apelqvist et al., 1999), not all cells lost Hes1 expression after Ptf1a\textsuperscript{-/-}-mediated deletion of Rbpj (Fujikura et al., 2007). It was proposed that the pancreatic buds contain different classes of progenitors with respect to their sensitivity to Rbpj-mediated Notch signaling and that other factors are involved in maintaining Hes1 expression. Recently, Sox9 was shown to be required for expression of Hes1 and normal proliferation of MPCs (Seymour et al., 2007), supporting the idea that Hes1 expression may be controlled by multiple inputs.

Here we demonstrate that endodermal Hes1 expression is independent of Dll1 function between E8.0 and E9.0 and that onset of Ptf1a expression around E9.0 directly activates Dll1 expression in MPCs. At this stage, Hes1 expression in MPCs becomes Dll1-independent. Accordingly, we demonstrate that the phenotypes of Hes1\textsuperscript{-/-} and Dll1\textsuperscript{-/-} embryos diverge at key points and that Hes1/Dll1 double mutants display an intermediate phenotype. Moreover, we find that early pancreatic hypoplasia in Dll1 mutants (Apelqvist et al., 1999) is independent of endocrine development and thus not caused by excessive endocrine differentiation and an associated depletion of progenitors. Instead, a reduced rate of proliferation in MPCs appears to be responsible. Unexpectedly, we find that Hes1 is required to sustain normal Ptf1a protein expression for a brief period during bud formation. Dll1 expression in turn also depends on Hes1, demonstrating that epistatically, Hes1 acts both up- and downstream of Dll1. Collectively, our results suggest that Hes1 initially has Notch-independent effects, or alternatively that Dll1 and Hes1 are involved in spatiotemporally distinct activities of the Notch pathway. Additionally, our data demonstrate that Ptf1a via activation of Dll1 stimulates MPC proliferation and contributes to Hes1 activation, and thereby may indirectly contribute to maintaining high Ptf1a protein levels.

### MATERIALS AND METHODS

#### Transgenic mouse lines

- **Dll1<sup>Min<sub>Gos</sub></sup> (Dll1<sup>lacZ</sup>-/-)** (Hrabe de Angelis et al., 1997), Tg(Hes1-EGFP)<sup>1Hri</sup><br>
  - BAC transgenic (Klinck et al., 2011), Neurog3<sup>1Hri</sup><br>
  - Tg(Ptf1a<sup>1Hri</sup>) (Wang et al., 2009), Ptf1a<sup>1Hri</sup><br>
  - Neurog3<sup>1Hri</sup> (Kawaguchi et al., 2002) and Hes1<sup>1Hri</sup> (Ishibashi et al., 1995) mouse lines were all kept as heterozygotes and genotyped by PCR and Southern blotting as previously described. All animal experiments described herein were approved by the Institutional Animal Welfare Committee at Hagedorn Research Institute and by the Danish Authorities for Animal Research.

#### Immunohistochemistry

Whole-mount immunofluorescent staining of embryos and dissected tissues was performed as previously described (Ahnfelt-Ronne et al., 2007b), except for the cleared Notch1 staining, where the specimens were pretreated by heating in 10 mM sodium citrate, pH 6 in a boiling water bath for 1 hour. Primary antibodies: rat anti-E-cadherin (R&D Systems), 1:1000; rabbit anti-\beta-galactosidase (MP Biomedicals), 1:1600 or 1:5000 with Tyramide Signal Amplification (TSA) (PerkinElmer, USA); goat anti-Pdx1 (Beta Cell Biology Consortium), 1:10,000; rabbit anti-Neurog3 (Ahnfelt-Ronne et al., 2007b) 1:16,000 with TSA; mouse anti-Nkx6-1 (Pedersen et al., 2006), 1:1000; rabbit anti-Cleaved Notch1 (Cell Signaling), 1:100 with TSA; rabbit anti-GFP (Living Colors, Clontech), 1:2000 with TSA; goat anti-Sox17 (R&D Systems), 1:1000; guinea pig anti-glucagon (Millipore), 1:10,000; rabbit anti-Ptf1a (Hald et al., 2008), 1:20,000 with TSA; rabbit anti-Sox9 (Millipore), 1:500 with TSA. The samples were cleared in BABB (benzyl alcohol:benzyl benzoate:1:2) before z-stack image scanning using a Zeiss LSM510 META Axio Imager connected to an LSM 510 laser module.

#### Chip-Seq analysis

Chromatin immunoprecipitation sequencing (ChIP-Seq) was performed as previously described (Hald et al., 2003). Primary antibodies: rabbit anti-\beta-galactosidase (MP Biomedicals), 1:1600; mouse anti-Neurog3 (Zahn et al., 2004), 1:500; goat anti-Pdx1 (Beta Cell Biology Consortium), 1:15,000; goat anti-GFP (Abcam), 1:1000; rabbit anti-Ptf1a (Hald et al., 2008), 1:4000.

**Neurog3** cell quantifications

E9.0-9.5 dorsal pancreas buds were z-stack image scanned with the Achromplan 20\times/0.5 W Ph2, WD 7.9 mm objective and Neurog3 positive cells were counted manually on every sixth section for 2.6 µm samples.

**Pancreatic bud volume quantifications**

Quantification of bud volume was based on Pdx1 immunoreactivity in whole-mount z-stacks and quantified using the Imaris X64 software version 7.1.1 (Bitplane AG, Zurich, Switzerland). Surface-rendering-area detail was set to 2 µm. Thresholding was set to absolute intensity and manually adjusted to cover the Pdx1-positive area of the proper pancreas buds (not including any duodenal or bile duct epithelium).

**BrdU labeling and quantification**

BrdU in saline suspension was injected at 100 µg/g intraperitoneally 1.5 hours before sacrifice. E10.5 embryos were fixed overnight in 4% formalin pH 7.0 and sectioned transversely at 200 µm on the vibratome. The sections were treated for 2 hours in 2 M HCl and subsequently stained using the protocol for whole-mount samples (see above). Primary antibodies: goat anti-Pdx1 (Beta Cell Biology Consortium), 1:10,000; rat anti-BrdU (Abcam), 1:500. After clearing in BABB, sections with dorsal pancreas were z-stack image scanned with the Plan-Neofluar 25\times/0.8, WD 0.21 mm objective and the number of Pdx1-positive and Pdx1-BrdU double-positive cells were counted manually on every fourth optical section, or the sections were z-stack image scanned with the Achromplan 20\times/0.5 W Ph2, WD 7.9 mm objective and counted manually on every second optical section.

### Whole-mount X-gal staining

E14.5 dissected Dll1<sup>lacZ</sup>-/- pancreas was fixed for 2.5 hours in 4% formalin pH 7.0 and stained as described (Lobe et al., 1999).

### RESULTS

#### Dll1, NICD and Hes1 expression in early endoderm

To test predictions of the classical lateral inhibition model we first used immunofluorescence (IF) to map the expression patterns of Dll1, NICD and Hes1 expression, relying on the \beta-galactosidase (\beta-gal) and EGFP reporters in Dll1<sup>Min<sub>Gos</sub></sup> (Dll1<sup>lacZ</sup>-/- hereafter) mice (Hrabe de Angelis et al., 1997) and Tg(Hes1-EGFP)<sup>1Hri</sup> BAC transgenic mice (Klinck et al., 2011), respectively. We found \beta-gal expression in presomitic mesoderm and nascent somites in E8.25 embryos (Fig. 1A) and in scattered cells of the dorsal pancreatic
endoderm of E9.0 *Dll1lacZ/+ embryos, whereas the ventral bud was negative (Fig. 1B). At E9.5, β-gal was expressed in Neurog3+ cells and uniformly in the dorsal bud epithelium but absent from the ventral bud as well as the surrounding duodenal endoderm (Fig. 1C), consistent with previous analyses of *Dll1* expression by in situ hybridization (Apelqvist et al., 1999; Bettenhausen et al., 1995). Notably, a few Neurog3+ cells were found in the lateral walls between the two pancreatic buds. Based on the timing of appearance, we speculate that these cells will join the dorsal glucagon cell cluster once differentiated. Alternatively, they may be the early precursors of enteroendocrine cells. At E10.5, β-gal expression was also found throughout the ventral bud epithelium (Fig. 1D) as well as in glucagon+ cells of the dorsal bud, possibly due to the long half-life of β-gal (not shown). β-gal expression was also seen in some endocrine precursors and in the developing acini at the secondary transition (supplementary material Fig. S1A-D).

Fig. 1. *Dll1*, NICD and *Hes1* expression in endoderm. (A-D) Optical sections of E7.5 to E10.5 *Dll1lacZ/+* embryos whole-mount stained for β-galactosidase, Cdh1, Pdx1, Neurog3 or Nkx6-1. The arrows in C point to Neurog3+ cells that co-express β-gal. The asterisk in D marks trapping of the anti-Nkx6-1 antiserum in the gut lumen. (E-I') Optical sections of E7.5 to E9.0 wild-type embryos whole-mount stained for NICD and Cdh1. The arrowheads in I and I' indicate the NICD expression in the dorsal pancreatic epithelium. (J-N') Optical sections of E7.5 to E9.0 Tg(*Hes1-EGFP)*^TRE^ embryos whole-mount stained for EGFP, Sox17, Cdh1 or Pdx1. The arrowheads in N point out Hes1-EGFP expression in the dorsal gut tube endoderm. (O-R) Cryosections of E10.5 *Dll1lacZ/+ Tg(*Hes1-EGFP)*^TRE^ double heterozygotes (O,P), Tg(*Hes1-EGFP)*^TRE^/Tg(*Dll1lacZ/+* (Q) and *Dll1lacZ/+* (R) embryos. (O) Double IF detection of *Ptf1a* and EGFP. (P) Double IF detection of β-galactosidase and EGFP. (Q) Double IF detection of Neurog3 and EGFP. (R) Double IF detection of β-galactosidase and Neurog3. The arrows in Q and R point to Neurog3+ cells. a, anterior; dp, dorsal pancreas; e, endoderm; fg, foregut; nt, neural tube; p, posterior; psm, presomitic mesoderm; vp, ventral pancreas. Scale bars: 50 μm. See also supplementary material Fig. S1.
NICD immunoreactivity was seen in definitive endoderm already at E7.5 (Fig. 1E), coinciding with onset of Hes1 expression (Fig. 1J). At E8.25, NICD and Hes1 were broadly expressed in anterior and posterior endoderm, including the prospective pancreatic regions (Fig. IF-H,K-M). From E9.0, NICD expression in the midgut region was confined to the dorsal pancreas bud (Fig. 1I, arrowheads) whereas Hes1 additionally was expressed in a stripe in the prospective dorsal midgut (Fig. 1N, arrowheads). Double IF for Ptf1a and Hes1-EGFP on Dll1lacZ/+; Tg(Hes1-EGFP)1Hri showed extensive overlap of expression (Fig. 1O), as did double IF for β-galactosidase and EGFP on an adjacent section (Fig. 1P), indicating that Ptf1a and Dll1 also display extensive overlap of expression. By contrast, most of the β-galactosidase+ and EGFP+ cells were negative for Neurog3 (Fig. 1Q,R), but the few Neurog3+ cells also appear positive for β-galactosidase and Hes1 at E10.5. However, the long half-lives of both of our reporters (EGFP and lacZ) make it difficult to firmly conclude true co-expression.

To determine if endodermal Notch activation and Hes1 expression depends on Dll1 activity, we analyzed NICD expression in wild-type and Dll1lacZ/lacZ embryos and EGFP expression in crosses of Tg(Hes1-EGFP)1Hri and Dll1lacZ/+ mice. NICD expression was reduced in E10.5 Dll1lacZ/lacZ embryos compared with controls (Fig. 2A,B), but appeared to recover, approaching wild-type levels at E11.5 (Fig. 2C,D). Hes1-EGFP expression was normal in E8.25 Dll1lacZ/lacZ embryos (Fig. 1E,I) but had partly disappeared from the dorsal pancreas endoderm at E9.5 (Fig. 1F,J) and was almost lost at E10.5. Remarkably, and coinciding with the reappearance of NICD, Hes1-EGFP expression was restored in E11.5 Dll1lacZ/lacZ embryos (Fig. 1H,L).

Ptf1a is required for Dll1 expression in MPCs

Although expression of Dll1 was observed in Neurog3+ cells as expected, we also noted expression in the remainder of the early dorsal pancreatic epithelium (Fig. 1C,D,P). It thus seems unlikely that Neurog3 can account for all Dll1 expression in the pancreatic anlage. To test this notion we crossed Dll1lacZ/+ onto a Neurog3tetTA/tetTA (Neurog3 null mutant) background and analyzed for β-gal expression. We found strong β-gal expression in E10.5 Dll1lacZ/+; Neurog3tetTA/+ MPCs (Fig. 3B,E), demonstrating that Dll1 expression in early MPCs is independent of Neurog3.

As basic helix-loop-helix (bHLH) proteins regulate Dll1 expression in other tissues (Ito et al., 2000; Ma et al., 1998; Nelson and Reh, 2008; Yoon and Wold, 2000), and as the onset of Dll1 expression in MPCs, as determined above, coincides with the onset of expression of the bHLH protein Ptf1a (Hald et al., 2008), we next tested if Ptf1a was required for Dll1 expression in MPCs by crossing Dll1lacZ/+ onto a Ptf1aCre/Cre (Ptf1a null mutant) background and analyzing β-gal expression. Remarkably, β-gal expression was lost from Pdx1-expressing E10.5

![Fig. 2. NICD and Hes1 expression in Dll1 mutants.](image)

(A-D) Optical sections of whole-mount IF-stained E10.5 and E11.5 Dll1+/+ and Dll1lacZ/lacZ embryos. The embryos were stained for Pdx1, NICD and glucagon. Note that NICD is reduced in E10.5 Dll1lacZ/lacZ embryos but recovers at E11.5. (E-L) Optical sections of Tg(Hes1-EGFP)1Hri mice on Dll1+/+ (E-H) or Dll1lacZ/lacZ (I-L) background. Endodermal EGFP expression is normal in E8.25 Dll1lacZ/lacZ mice but becomes reduced beginning at E9.5 and is essentially lost at E10.5. However, EGFP expression recovers at E11.5 coincident with NICD recovery. dp, dorsal pancreas; e, endoderm; fg, foregut; psm, presomitic mesoderm; vp, ventral pancreas. Scale bars: 50 μm.
Ptf1a induces Dll1 in pancreas

Dll1lacZ/Ptf1aCre/Cre pancreatic epithelial cells, but was retained in the endocrine lineage (Fig. 3C,F). It is possible that the loss of Dll1 expression is a secondary effect of an overall cell fate change due to the absence of Ptf1a, but as Pdx1 is still expressed it is more likely that Ptf1a is required for Dll1 expression in MPCs independently of Neurog3 and the formation of endocrine progenitors in the early pancreas.

We then used ChIP-Seq analysis to test if Ptf1a could bind directly to regulatory sequences in the Dll1 gene. As cell numbers are limited in E9.5 buds, we chose E15.5 pancreas where Ptf1a and Dll1 are co-expressed in developing acinar cells (not shown). As Ptf1a binds DNA together with Rbpj in early MPCs and Rbpjl in later acinar cells (Beres et al., 2006; Masui et al., 2007), we used antibodies against Ptf1a and Rbpj to perform the ChIP-Seq. We found that both proteins bound to the proximal promoter region of Dll1 as well as to three or four sites in a conserved upstream region located from –4.7 to –6.4 kb upstream of the transcriptional start site (Fig. 3G). The peaks were discrete, present at other known Ptf1a-dependent regulatory sites (e.g. the Ptf1a enhancer, the Rbpjl promoter and Cpa1), statistically significant, and rare compared to the size of the genome (supplementary material Fig. S2A). Inspection of the nucleotide sequence revealed potential binding sites matching the consensus sequence (Beres et al., 2006) for the Ptf1a-Rbpj complex (supplementary material Fig. S2B). Although
these proposed binding sequences diverge somewhat from the consensus of either the E-box or the TC-box motifs (supplementary material Fig. S2B), this is not uncommon for bone fide binding sites of the Ptf1 complex (Beres et al., 2006; Masui et al., 2010). Together with the loss of Dll1 expression in E10.5 Ptf1a mutant epithelium, these observations suggest that a Ptf1a-Rbpj complex might bind to Dll1 regulatory sequences in early MPCs.

**Dll1 is required for continued formation of endocrine precursors**

The relevance of the classical lateral inhibition model was based on the previously reported similarity of Dll1, Rbpj and Hes1 mutant phenotypes (Apelqvist et al., 1999; Jensen et al., 2000b) and the known role of homologs of these genes in Drosophila neuroblast specification where single cell suppression of neighbors via lateral inhibition is well documented (Simpson, 1990). However, the uncoupling of Dll1 and Hes1 expression and the Neurog3 independent regulation of Dll1 expression demonstrated above conflicts with predictions made by this canonical lateral inhibition model. Moreover, the different genetic backgrounds on which original analyses of Dll1 and Hes1 mutants were conducted might lead to differences in the extent and timing of precocious endocrine differentiation. This prompted us to reexamine the pancreatic phenotypes of Dll1 and Hes1 mutants on the same genetic background (CD1) and to examine Dll1/Hes1 double mutants in order to determine the epistatic relationship between these two genes during early pancreas development.

We collected E8.5 to E12.5 wild-type and mutant embryos and analyzed the expression of Pdx1, Neurog3 and glucagon to view the pancreatic epithelium, endocrine precursors and differentiated endocrine cells, respectively, the latter predominantly expressing glucagon at these early stages (Jorgensen et al., 2007). To ensure sufficient sampling of all the tissue involved, we used whole-mount IF and high-resolution confocal microscopy (Ahnfelt-Ronne et al., 2006) to obtain a global 3D view of the distribution of labeled cells. We found that Hes1 (but not Dll1) mutants showed increased numbers of Neurog3+ cells in dorsal endoderm as early as eight somites (~E8.25), just around the onset of dorsal Pdx1 expression and emergence of the earliest Neurog3+ cells (Fig. 4A-C). At the 13-20 somite stage (~E9.0) we found a prominent increase in the number of Neurog3+ cells in both Dll1 and Hes1 mutants as well as in Dll1/Hes1 double mutants (Fig. 4E-H). However, while 21-28 somite (~E9.5) Hes1 mutants still displayed increased numbers of Neurog3+ cells compared with wild type, Dll1 mutants showed a dramatic decrease in the number of Neurog3+ cells (Fig. 4L), indicating that new Neurog3+ cells were not formed given that Neurog3 is transiently expressed. This finding conflicts with that of Edlund and coworkers, who found increased numbers of Neurog3+ cells at E9.5 (Apelqvist et al., 1999). We suspect that this conflict may be caused by the different genetic backgrounds that may cause a minor difference in the timing of precocious endocrine differentiation. Notably, Dll1/Hes1 double mutants showed an intermediate phenotype with a mild excess of Neurog3+ cells. Remarkably, the dorsal bud epithelium of double mutants appeared to show a depletion in Neurog3+ cells, whereas excess Neurog3+ cells were found in the lateral gut wall (Fig. 4L), a phenotype also seen in Dll1 mutants heterozygous for Hes1, although the number of Neurog3+ cells is smaller in these embryos (not shown). Quantification of Neurog3+ cells confirmed that Dll1, Hes1 and Dll1/Hes1 mutant embryos had an equal increase of Neurog3+ cells at the 13-20 somite stage and that Dll1 mutants specifically showed a depletion of Neurog3+ cells at the 21-28 somite stage (Fig. 4M). The mild excess of Neurog3+ cells in double mutants is most likely the result of excess Neurog3+ cells forming in the lateral gut wall combined with a depletion of Neurog3+ cells in the dorsal bud itself.

Using the CD1 background, we could obtain living Dll1 mutants until E12.5. At E11.0 the pancreatic epithelium was smaller than normal, as previously noted (Apelqvist et al., 1999). Neurog3+ cells were far fewer, and this effect was maintained in E12.5 Dll1 mutants (supplementary material Fig. S3). In contrast, Hes1 mutants had numerous Neurog3+ cells at these stages (supplementary material Fig. S3). Notably, from E10.5 onwards we found aberrant dorsal bud morphogenesis in Hes1 mutants, a defect that may be associated with the ectopic pancreas found later in these animals (Fukuda et al., 2006; Sumazaki et al., 2004). We conclude that the Notch ligand Dll1 but not the Notch signaling effector Hes1 is required for the continued formation of Neurog3+ endocrine precursors.

**Ptf1a-mediated Dll1 expression is required for MPC proliferation**

Dll1 mutants have hypoplasia of the Pdx1+ epithelium at E10.5, ostensibly caused by excessive differentiation and consequent depletion of Pdx1+ progenitors (Apelqvist et al., 1999). However, the ventral bud is also reduced in size despite the absence of excessive endocrine differentiation (Fig. 5C), suggesting that another mechanism is responsible for the reduced size of the Pdx1+ epithelium. If excessive endocrine development is causing the hypoplasia, then elimination of endocrine development should counteract it. To test this notion directly, we quantified dorsal and ventral bud sizes in E10.5 Dll1lacZNeurog3TgTA/TA (double null) embryos, where endocrine development was expected to be abolished due to the absence of Neurog3 (Gradwohl et al., 2000). On a Neurog3 wild-type background, we found that dorsal and ventral bud volume was reduced ~5-fold in Dll1lacZlacZ mutants compared with controls (Fig. 5A,C,G). Remarkably, a similar fold reduction was observed in Dll1lacZNeurog3TgTA/TA double null embryos, in spite of a drastic reduction of endocrine differentiation (Fig. 5F,G). Notably, Neurog3TgTA/TA embryos showed a modestly reduced dorsal bud size compared to wild types. The magnitude is, however, not sufficient to confound our conclusion about a role for Dll1 in epithelial growth independent of its inhibitory role in endocrine differentiation. Moreover, ventral bud size is not changed in Neurog3TgTA/TA embryos consistent with the very limited differentiation at this stage of ventral bud development. Nevertheless, ventral bud size is equally reduced in Dll1lacZNeurog3TgTA/TA double null embryos (Fig. 5G), bolstering the notion of a Dll1 function independent of its inhibition of endocrine differentiation.

Surprisingly, a few endocrine cells were observed in the Dll1 null and heterozygote background (Dll1lacZNeurog3TgTA/TA and Dll1lacZNeurog3TgTA/TA embryos) compared with the complete loss of the endocrine lineage in Dll1lacZNeurog3TgTA/TA embryos (Fig. 5D-F; data not shown). We speculate that this may be caused by derepression of other bHLH genes that could substitute for Neurog3 and inefficiently initiate the endocrine commitment process on the Dll1 mutant background. Nevertheless, the great reduction of endocrine differentiation should be sufficient to restore pancreatic epithelial development in Dll1 mutants if depletion of progenitors was the cause of the hypoplasia. Notably, Dll1lacZlacZ heterozygotes also showed reduced dorsal bud volume, on both wild-type and Neurog3 deficient backgrounds (Fig. 5G), demonstrating Dll1 haploinsufficiency.
Fig. 4. *Dll1* is required for continued formation of endocrine precursors. (A-C) Image stack projections of E8.25 wild-type, Dll1 and Hes1 mutant embryos whole-mount stained for Pdx1 and Neurog3. The inset in C shows an optical section of emerging Pdx1+ cells in the dorsal endoderm. Note that A and C are ventral views, whereas B is tilted more laterally. (D) Schematics of E8.25 and E9.0-9.5 embryos with red boxes indicating imaged areas in the other panels. (E-H) Image stack projections of E9.0 wild-type, Dll1, Hes1 and Hes1/Dll1 mutant embryos whole-mount stained for Pdx1 and Neurog3. The arrowheads in G point to ectopic expression of Neurog3 in the dorsal gut epithelium posterior to the Pdx1+ pancreas area. (E'-H') Optical sections through the dorsal bud region of the embryos shown in E-H. (I-L) Image stack projections of E9.5 wild-type, Dll1, Hes1 and Hes1/Dll1 mutant embryos whole-mount stained for Pdx1, Neurog3 and glucagon. (I'-L') Optical sections through the dorsal bud region of the embryos shown in I-L. (M) Bar graph showing quantification of Neurog3+ cells in E9.0 (13-20 somite) and E9.5 (21-28 somite) wild-type, Dll1, Hes1 and Hes1/Dll1 mutant embryos. Data are represented as mean ± s.d. #, P<0.001; *, P<0.002; †, P<0.005; by Student's t-test (compared with wild type); n=3-9. See also supplementary material Fig. S3.
Previous work ruled out apoptosis as a likely cause of pancreas hypoplasia in early \textit{Dll1} mutants (Apelqvist et al., 1999), and forced activation of Notch signaling in pancreatic endoderm induces or maintains progenitor proliferation (Ahnfelt-Ronne et al., 2007a; Hald et al., 2003; Murtaugh et al., 2003). We therefore measured BrdU incorporation and found that it is reduced in \textit{Dll1lacZ/lacZ} and \textit{Hes1\textsuperscript{--/--}} MPCs compared with wild-type controls (Fig. 5H,I), indicating that the hypoplasia seen in \textit{Dll1} mutants is caused by reduced proliferation of MPCs. Moreover, the persistence of severe hypoplasia in \textit{Dll1lacZ/lacZNeurog3\textsuperscript{TA/TA}} double null embryos together with only moderate hypoplasia in \textit{Neurog3} null embryos suggests that Ptf1a-induced \textit{Dll1} expression is required for normal proliferation of MPCs.

**Hes1 is required for normal Ptf1a expression in MPCs**

A typical feature of the interactions between Notch signaling and the bHLH proteins that activate Notch ligand expression is a negative feedback loop in which activation of Notch signaling represses the expression and/or function of the cognate bHLH protein. Indeed, a previous report has shown that Hes1 can inhibit...
the later ‘acinar-differentiation-promoting’ function of Ptf1a (Esni et al., 2004), and co-immunoprecipitation and yeast two-hybrid experiments have suggested that this inhibition might be mediated via a direct Hes1-Ptf1a protein-protein interaction (Ghosh and Leach, 2006). Nevertheless, Ptf1a activity is required in MPCs (Kawaguchi et al., 2002), and the concurrent expression with Hes1 seems to rule out that it inhibits only Ptf1a function at this earlier stage. In order to gain a better understanding of Hes1-Ptf1a interactions in early MPCs, we analyzed Ptf1a expression by whole-mount IF in E9.5 Hes1<sup>−/−</sup> embryos. Remarkably, we found that Ptf1a protein is lost from most of the dorsal (but not ventral), Pdx1<sup>+</sup> MPCs in Hes1<sup>−/−</sup> embryos compared with wild-type controls (Fig. 6A,B). By contrast, expression of Sox9, which is believed to act upstream of Hes1 (Seymour et al., 2007), was unaffected by the loss of Hes1 (Fig. 6C,D). The loss of Ptf1a protein in Hes1<sup>−/−</sup> embryos was also noticeable at E10.5 but had recovered by E12.5 (supplementary material Fig. S4).

The loss of Ptf1a protein in E9.5-10.5 Hes1<sup>−/−</sup> dorsal bud MPCs raises an intriguing possibility: as Dll1 expression is dependent on Ptf1a at this stage (see above), one would predict that Dll1 expression should be affected by the loss of Hes1, leading to an epistatic relationship between Dll1 and Hes1 that is in fact the opposite of that predicted by the classical lateral inhibition model. To test this notion, we compared lacZ expression in E9.5 and E10.5 Dll1lacZ<sup>+/−</sup>Hes1<sup>−/−</sup> and Dll1lacZ<sup>+/+</sup>Hes1<sup>+</sup> embryos. Remarkably, and as predicted by the loss of Ptf1a expression, β-gal expression was

Fig. 6. Hes1 is required for normal Ptf1a and Dll1 expression in MPCs. (A-D′) Image stack projections of whole-mount stained E9.5 wild-type (A,C) and Hes1<sup>−/−</sup> (B,D) embryos, showing expression of Pdx1, glucagon and Ptf1a (A,B) or Sox9 (C,D). A′-D′ show the green channel alone to give an unobstructed view. Note the loss of Ptf1a immunoreactivity in Hes1<sup>−/−</sup> dorsal bud. (E-H′) Image stack projections of whole-mount stained E9.5 (E,F) and E10.5 (G,H) wild-type (E,G) and Hes1<sup>−/−</sup> (F,H) embryos, showing expression of Dll1 (β-gal), Pdx1 and glucagon. E′-H′ show the red channel alone to give an unobstructed view. Note the reduced levels of β-gal expression in E9.5 and E10.5 dorsal buds and E10.5 ventral buds in Hes1 mutants. The asterisks in E mark specks of non-specific fluorescence from the secondary antibody. dp, dorsal pancreas; vp, ventral pancreas. Scale bars: 50 μm. See also supplementary material Fig. S4.
reduced in E9.5 Dll1lacZ−/− Hes1+/− dorsal bud MPCs compared with Dll1lacZ+/− Hes1+/− controls (Fig. 6E,F). However, in the ventral bud, which normally does not express Dll1-lacZ at this stage (Fig. 1C and Fig. 5E), we observed the appearance of scattered β-gal+ cells in Dll1lacZ+/− Hes1+/− embryos (Fig. 6F), presumably owing to the precocious appearance of Dll1-expressing, Neurog3+ endocrine progenitors in the Hes1 mutant background. In addition, in addition to the loss of Dll1-lacZ from E10.5 dorsal buds, the normal onset of Dll1-lacZ expression in the ventral bud MPCs at this time is lost in Dll1lacZ−/− Hes1+/− embryos (Fig. 6G,H). Thus, Hes1 is required for normal levels of Dll1 expression in MPCs.

**DISCUSSION**

Based on the findings reported here, we propose that Ptf1a is a crucial transcriptional activator of Dll1 expression, and therefore also of Notch signaling, in multipotent pancreatic progenitor cells. As a consequence of this mode of Notch activation, our results refute the classical lateral inhibition model proposed a decade ago (Apelqvist et al., 1999; Edlund, 2002; Jensen et al., 2000a; Skipper and Lewis, 2000). Our results also demonstrate a hitherto unsuspected requirement for Hes1 in the maintenance of high Ptf1a protein levels and as a result also for normal Dll1 expression. Thus, Hes1 acts upstream of Dll1 in addition to its role downstream of Dll1.

**Ptf1a-mediated activation of Dll1 reveals a broader role for Dll1 than classical lateral inhibition**

We and others have previously provided evidence that Notch signaling, via its potential downstream target Hes1, regulates pancreatic endocrine development (Apelqvist et al., 1999; Jensen et al., 2000b). The classical lateral inhibition model puts forward that Neurog3-expressing endocrine progenitors signal to neighboring cells via Dll1 and instruct these to adopt alternate fates (e.g. pancreatic exocrine or persistence as MPCs). Lateral inhibition was proposed as the mechanism that coupled Notch signaling and Hes1 expression to the regulation of Neurog3 expression and endocrine differentiation. Nevertheless, more recent studies found no change in the number of Neurog3+ cells in Ptf1aCre−/− conditional Notch1-2-deficient embryos, while confirming that endocrine development was disturbed in conditional Rbpj knockouts (Nakhai et al., 2008). It was proposed that the requirement for Notch signaling in pancreatic development was less important than previously thought and that Rbpj may act independently of its role as a Notch effector. However, a requirement for Notch signaling during early pancreas development was not ruled out.

The results presented here demonstrate that Notch signaling is indeed essential during early, bud-forming stages of pancreatic development, from E8.0 to at least E10.5. Importantly, our observations on (1) the timing, extent and interdependency of Dll1 and Hes1 expression, (2) the Ptf1a-dependent expression of Dll1 in MPCs, (3) the discordant phenotypes of Dll1 and Hes1 mutants, (4) the pancreatic hypoplasia of Dll1/Neurog3 double null mutants and (5) the transient loss of Ptf1a protein and, in turn, Dll1 expression in Hes1+/− MPCs, challenge the classical view of the lateral inhibition model. For example, Hes1 is expressed in definitive endoderm (DE), long before Dll1 becomes expressed in DE. Even so, the presence of NICD in E7.5 DE suggests that Notch signaling is active and that this may be responsible for activating Hes1 expression. Anteriorly, Dll1 does not appear to be expressed at the right time and place to activate Notch in foregut DE, and we are not aware of other Notch ligands that are. Posteriorly, Dll1 and Dll3 is expressed in the adjacent presomitic mesoderm (Geffers et al., 2007), but it is not clear if Notch ligands expressed in presomitic mesoderm can signal across the basal lamina to activate Notch in DE. Nonetheless, Notch is activated and its target Hes1 is expressed broadly in both anterior and posterior DE, but not via Neurog3-induced Dll1 expression. Indeed, Dll1-independent Hes1 expression is evidenced by the continued expression of Hes1-GFP in Dll1 mutant MPCs until E9.5. Later, at E10.5, both NICD and Hes1-GFP expression disappears from Dll1 mutant dorsal bud epithelium, showing that Hes1 expression becomes dependent on Dll1 at this stage. The recovery of both NICD and Hes1-GFP expression in E11.5 Dll1 mutants may be due to onset of Jag1 expression around this time (Apelqvist et al., 1999) or due to the expression of non-canonical Notch ligands Dlk-1 (Carlsson et al., 1997) and/or Dner (J. Hald, personal communication) in the pancreatic epithelium around this time. While the lateral inhibition model correctly predicts Neurog3-dependent Dll1 expression in endocrine progenitors, it fails to predict our observation of Dll1 expression in MPCs as well as this expression being independent of Neurog3. The Ptf1a-dependent expression of Dll1 in MPCs is likely to be through direct binding to conserved binding sites in the Dll1 5′-flanking region. Thus, Neurog3 and Ptf1a activate Dll1 in endocrine progenitors and MPCs, respectively. A model incorporating the novel relationships between these transcription factors and Notch signaling components is presented in Fig. 7.

**Dll1 is required for continued formation of endocrine progenitors**

Refuting the classical lateral inhibition model prompted us to reexamine the pancreatic phenotypes resulting from deleting these genes, and to generate and examine Dll1/Hes1 double mutants in order to elucidate the epistatic relationship between Dll1 and Hes1. Overall, our results from these analyses are in agreement with previous studies but importantly, they reveal several features not previously appreciated that differ between the two mutants. First, the endocrinogenic phenotype of Hes1 mutants is evident before the onset of Dll1 expression in the pancreatic endoderm and before Dll1 mutants show signs of endoderm development, supporting the idea that Hes1 is acting independently of Dll1 at this stage. Both single mutants and the double mutant show an equal increase in the number of Neurog3+ cells at ~E9.0, which is similar in magnitude to that previously observed in the Dll1 mutant by Apelqvist and coworkers (Apelqvist et al., 1999), but occurring half a day earlier than reported by this group. This difference in timing could be related to the different genetic backgrounds used.

From our 3D projections of whole embryos, it is evident that many of the Neurog3+ cells found in Hes1 mutants are located in the Pdx1+ lateral gut walls between the dorsal and ventral pancreatic buds, while they are confined to the dorsal bud in Dll1 mutants. Moreover, Hes1 mutants show ectopic Neurog3+ cells in the dorsal part of the prospective duodenum, exactly where we observe Hes1-GFP expression. Importantly, neither the lateral walls between the pancreatic buds nor the dorsal duodenum express Dll1 (consistent with the lack of Ptf1a expression in these regions), showing that Hes1 can act independently of Dll1 not only dependent on the developmental stage but also depending on the exact location of the cells in the midgut region. The reduction of Neurog3+ cells in E9.5 Dll1 mutants stands in contrast to Hes1 mutants, which maintain the increase seen at E9.0, and Dll1/Hes1 double mutants, which show an intermediate phenotype with respect to Neurog3+ cell numbers. Inspection of 3D projections
revealed that the intermediate phenotype is related to the predominant location of endocrine precursors in the lateral gut walls in E9.5 Dll1/Hes1 double mutants, with the dorsal bud being essentially devoid of Neurog3+ cells as is also seen in E9.5 Dll1 single mutants.

The loss of endocrine precursors in Dll1 mutants has not previously been appreciated and is maintained until E12.5, the latest stage we could recover live embryos from. Loss of Neurog3+ cells from E11.5, after an initial increase at E10.5, has been reported in conditional Rbpj mutants (Fujikura et al., 2006; Nakhai et al., 2008). However, it has been unclear if this is a consequence of compromised Notch signaling or the result of disrupting the function of the Ptf1a/Rbpj transcription factor complex (Fujikura et al., 2006; Nakhai et al., 2008). The similarity of Dll1 and Rbpj mutant phenotypes in relation to Neurog3+ cell numbers is consistent with the notion of loss of Neurog3+ cells being due to loss of Notch signaling. Still, other explanations cannot be ruled out, and the cellular mechanism behind the depletion of Neurog3+ cells remains unresolved. It has been proposed that an endocrine-restricted pool of progenitors adopt the endocrine fate prematurely when Notch signaling is compromised, resulting in depletion of this pool specifically while allowing continued development of exocrine progenitors (Fujikura et al., 2006; Nakhai et al., 2008). If this is the case, then Hes1 must act at least partly independent of Notch, as Hes1 mutants have increased Neurog3+ cells at least until E12.5.

An alternative explanation for the loss of endocrine precursors in Dll1 mutants can be envisioned if one assumes that inhibitory Dll1-Notch interactions are required in cis for initiating and/or maintaining the endocrine differentiation program. Recent work has shown that cis-interactions between Notch and its ligands are crucial for preventing signal sending cells from receiving reciprocal signaling from their neighbors (Sprinzak et al., 2010). This notion offers an attractive mechanism for allowing formation of endocrine precursors during early pancreas development. We show here that Dll1 expression in neighboring cells is under control of Ptf1a and not Neurog3. Thus, the regulatory logic from the classical lateral inhibition scheme, where high levels of Dll1 in the differentiating cell act via Notch to downregulate Neurog3 and in turn Dll1 in neighboring cells, breaks down. However, high levels of Neurog3-induced Dll1 might act in cis to prevent signaling to the differentiating cells from Ptf1a-induced Dll1 expression in neighboring MPCs, and thus allow differentiation to proceed in spite of continued Dll1 expression in adjacent MPCs. In such a model, endocrine precursors can form in Dll1 mutants as long as no other Notch ligand is expressed in the MPCs (e.g. as seen in E9.0 Dll1 mutants), but would be prevented when other Notch ligands become expressed in MPCs or if Notch becomes activated independent of canonical ligand expression. Further experiments determining (1) the onset of expression of other canonical and non-canonical Notch ligands and (2) whether conditional deletion of Dll1 in endocrine precursors prevents their differentiation are needed to test this model.

**Ptf1a-mediated Dll1 expression contributes to MPC proliferation and to maintaining Ptf1a protein levels**

Ptf1a-induced Dll1 expression in MPCs raises the question of whether this expression has a function besides preventing premature endocrine development. Previous work has suggested that Notch signaling stimulates MPC proliferation (Ahnfelt-Ronne et al., 2007a; Hald et al., 2003; Murtaugh et al., 2003), possibly via Hes1-mediated repression of p57 expression (Georgia et al., 2006), and we show here that fewer Dll1 (and Hes1) mutant MPCs incorporate BrdU than seen in wild-type controls, and that prevention of endocrine differentiation by eliminating Neurog3 fails to restore the reduced bud size in Dll1 mutants. Together these results suggest that the early pancreatic hypoplasia seen in Dll1 mutants is caused by reduced proliferation of MPCs, rather than by depletion of MPCs by premature endocrine differentiation. It would thus seem that MPCs may exist in simultaneous sending and receiving states, contradicting the notion of these states being mutually exclusive (Sprinzak et al., 2010).

Ptf1a-dependent Dll1 expression also raises the question about potential feedback regulation of Ptf1a itself. Loss of Ptf1a protein from most Hes1−/− dorsal bud MPCs, shows that Ptf1a transcription or Ptf1a protein expression depends on Hes1, at least briefly. Moreover, as we showed Dll1 expression to be Ptf1a dependent, we could accurately predict that Dll1 expression was reduced in Hes1−/− embryos. The mechanism behind this transient loss of Ptf1a remains obscure, but we speculate that potential protein-protein interactions between Ptf1a and Hes1 (Ghosh and Leach, 2006) may be involved. It is possible that Ptf1a and Hes1 are both part of a larger protein complex and that disruption of this complex in Hes1 mutants destabilizes Ptf1a. Surprisingly, this places Dll1 downstream of Hes1 in addition to its role upstream of Hes1 and suggests that Ptf1a, via Dll1, is contributing to Hes1 activation and thereby may indirectly contribute to maintaining high Ptf1a protein levels.

In the present work we have exclusively analyzed early pancreatic development. Recent findings suggest even further complexity in Notch-mediated regulation of later pancreatic endocrine development (Cras-Meneur et al., 2009; Golson et al., 2009). Regardless of these later complexities in Notch-mediated regulation of pancreatic development, our results demonstrate that early pancreas development relies on Dll1-mediated Notch signaling to maintain normal proliferation of MPCs and to restrict early endocrine development by a mechanism that differs from the classical lateral inhibition model. These findings may have direct relevance for Notch-mediated control of endocrine differentiation in other parts of the gastrointestinal tract.

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**Fig. 7. Model of the relationships between transcription factors and Notch signaling components in primary transition pancreas.** In contrast to the simple lateral inhibition model, the new model features Ptf1a-mediated activation of Dll1, which is crucial for Notch activation in MPCs. Furthermore, the model proposes that Notch is subject to cis-inhibition by high levels of Dll1 induced by Neurog3. Activated components of the regulatory system are shown in black, whereas components held inactive are gray.
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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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