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A Notch-dependent molecular circuitry initiates pancreatic endocrine and ductal cell differentiation

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SUMMARY

In the pancreas, Notch signaling is thought to prevent cell differentiation, thereby maintaining progenitors in an undifferentiated state. Here, we show that Notch renders progenitors competent to differentiate into ductal and endocrine cells by inducing activators of cell differentiation. Notch signaling promotes the expression of Sox9, which cell-autonomously activates the pro-endocrine gene *Ngn3*. However, at high Notch activity endocrine differentiation is blocked, as Notch also induces expression of the *Ngn3* repressor Hes1. At the transition from high to intermediate Notch activity, only Sox9, but not Hes1, is maintained, thus de-repressing *Ngn3* and initiating endocrine differentiation. In the absence of Sox9 activity, endocrine and ductal cells fail to differentiate, resulting in polycystic ducts devoid of primary cilia. Although Sox9 is required for *Ngn3* induction, endocrine differentiation necessitates subsequent Sox9 downregulation and evasion from Notch activity via cell-autonomous repression of Sox9 by Ngn3. If high Notch levels are maintained, endocrine progenitors retain Sox9 and undergo ductal fate conversion. Taken together, our findings establish a novel role for Notch in initiating both ductal and endocrine development and reveal that Notch does not function in an on-off mode, but that a gradient of Notch activity produces distinct cellular states during pancreas development.

KEY WORDS: Hes1, Ngn3, Notch, Sox9, Cyst, Pancreas, Mouse

INTRODUCTION

A fundamental question in tissue and organ development is how cells are coordinately instructed to move from a pluri- or multipotent state into differentiated states characteristic of a tissue or organ. Specifically, it is unknown how cues for maintaining multipotency are integrated with lineage-specific differentiation programs.

During pancreas development, three distinct cell types, namely acinar, ductal and endocrine cells, emerge from a domain of multipotent pancreatic progenitors in the early pancreatic epithelium (Gu et al., 2002; Kawaguchi et al., 2002). This domain subsequently resolves into a pre-acinar domain in the tips of the branching organ and a centrally located bipotential ductal/endocrine progenitor domain (Schaffer et al., 2010; Solar et al., 2009; Zhou et al., 2007). In mice, the majority of endocrine cells differentiate from this bipotential domain between embryonic day (E) 13.5 and E16.5, a time window coined the secondary transition. Still, the molecular mechanisms orchestrating the coordinated emergence of endocrine and ductal cells from this progenitor domain are virtually unknown.

Accumulating evidence suggests that Notch signaling plays a crucial role in this process. Loss-of-function studies have shown that the absence of Notch signaling components results in premature endocrine cell differentiation (Ahnfelt-Ronne et al., 2011; Apelqvist et al., 1999; Jensen et al., 2000); conversely, forced expression of the Notch intracellular domain (NICD) blocks endocrine cell formation (Greenwood et al., 2007; Hald et al., 2003; Murtaugh et al., 2003). The Notch signaling target Hes1 appears to play an important role in this process, as it directly binds and represses *Neurog3* (*Ngn3*), a gene necessary and sufficient for endocrine cell differentiation (Gradwohl et al., 2000; Gu et al., 2002; Lee et al., 2001). If Ngn3 levels are reduced or Ngn3 is absent, secondary transition progenitors adopt a ductal fate (Beucher et al., 2011; Magenheim et al., 2011; Wang et al., 2009). Whether Notch is required for ductal differentiation is still unclear. Based on the observation that certain ductal markers are still expressed in pancreata deficient for the Notch effector Rbpj (Fujikura et al., 2006; Fujikura et al., 2007), it has been proposed that Notch promotes duct formation indirectly by preventing differentiation into other cell types (Greenwood et al., 2007). Thus, the current view is that Notch blocks differentiation programs and thereby maintains progenitors in an undifferentiated state.

Still debated is the mechanism through which Notch controls endocrine cell formation. Paralleling established roles for Notch in neuronal development (Kageyama and Ohtsuka, 1999), Notch has been proposed to control *Ngn3* non-cell-autonomously via a lateral inhibition mechanism. Consistent with this notion, $Ngn3^+$ and $Hes1^+$ cells are found in a mosaic pattern throughout the epithelial cords (Ahnfelt-Ronne et al., 2007; Esni et al., 2004). Furthermore, analysis of *Ngn3*-deficient mice suggests that Hes1 expression is, at least partially, regulated by lateral inhibition from $Ngn3^+$ cells (Magenheim et al., 2011). However, some aspects of the *Dll1* and *Ngn3* mutant phenotypes are also discordant with the lateral inhibition model (Ahnfelt-Ronne et al., 2011; Wang et al., 2009), suggesting that Notch might have multiple functions in the developing pancreas.

We have shown previously that *Sox9* is required to maintain multipotent progenitors of the early pancreatic epithelium (Seymour et al., 2007). During the secondary transition, Sox9 is expressed in the bipotential ductal/endocrine epithelial cords, where it controls Ngn3 and Pdx1 expression (Dubois et al., 2011).

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Although the domains of Sox9 and Ngn3 partially overlap, Sox9 is largely excluded from Ngn3^{high} cells and is restricted to ducts in the adult pancreas (Seymour et al., 2008; Seymour et al., 2007). Although upstream regulators of *Sox9* in pancreas are still unknown, Sox9 expression has been shown to depend on Notch signaling in developing bile ducts and neurons (Taylor et al., 2007; Zong et al., 2009).

In this study, we explored a possible link between Notch and Sox9 in pancreatic endocrine and ductal differentiation. We show that Notch activity coincides with Sox9 and that Sox9 expression is controlled by Notch. Furthermore, we demonstrate that Sox9 is necessary for endocrine and ductal cell differentiation. Thus, by regulating Sox9, Notch renders progenitors bipotential for adopting endocrine and ductal fates.

MATERIALS AND METHODS

Mouse strains

The following mouse strains were utilized in this study: *Sox9^{flox}* (Kist et al., 2002), *Sox9-eGFP* (Gong et al., 2003), *Pdx1-Cre, Pdx1-CreER* (Gu et al., 2002), *Rosa26-CreER* (Vooijs et al., 2001), *Ngn3-Cre* (Schonhoff et al., 2004), *Ngn3^{eGFP}* (Lee et al., 2002), *Rosa26-eYFP* (Srinivas et al., 2001), *Rosa26-NotchIC* (Murtaugh et al., 2003) and *Pdx1-Ngn3ER-IRES-nGFP* (Johansson et al., 2007). Tamoxifen (Sigma) was dissolved in corn oil and administered by intraperitoneal (i.p.) injection. Midday on the day of vaginal plug appearance was considered to be E0.5.

Immunohistochemistry and cell quantification

Tissue was prepared and immunofluorescence staining performed as previously described (Seymour et al., 2008). Primary and secondary antibodies are listed in supplementary material Table S1. ApoTome images were captured on a Zeiss Axio-Observer-Z1 microscope with Zeiss AxioVision 4.8 and figures prepared using Adobe Photoshop/Illustrator CS4.

Entire embryonic pancreata were sectioned at 7-10 μm. Four evenly spaced sections per embryo and at least three embryos per each genotype were analyzed. To quantify the number of lineage-labeled Sox9$^+$, Ngn3$^+$, endocrine, acinar and ductal cells, all Sox9$^+$, Ngn3$^+$, hormone$^+$, amylase$^+$ or DBA$^+$ cells on a section were counted and the percentage expressing eYFP determined. A total of 19,436 and 13,300 eYFP$^+$ cells were counted in *Rosa26-eYFP; Pdx1-CreER* and *Sox9$^{flox/flox}$; Rosa26-eYFP; Pdx1-CreER* pancreata, respectively. In *Ngn3$^{+/eGFP}$* and *Ngn3$^{eGFP/eGFP}$* embryos, all eGFP$^+$ cells (2540 in *Ngn3$^{+/eGFP}$* and 2383 in *Ngn3$^{eGFP/eGFP}$* embryos) on a section were counted and the fraction of Sox9$^+$/eGFP$^+$, Hes1$^+$/eGFP$^+$ and Spp1$^+$/eGFP$^+$ determined. In HepG2 cell line, all eGFP$^+$ cells (164 in controls and 147 in *pCMV-Ngn3-IRES-eGFP*-transfected cells) on ten randomly selected microscope fields (200×) were counted and the percentage of Sox9$^+$ cells expressing eGFP determined. Cell counting was performed using Volocity 6.0.1 (PerkinElmer) (Fig. 3S) or manual analysis using Adobe Photoshop CS4 (Fig. 4F, Fig. 5O).

To detect nuclear expression of NICD2, 7 mm-thick paraffin sections were de-paraffinized followed by sequential re-hydration. Sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 minutes and cooled down for 45 minutes at room temperature (RT). After blocking with TSA blocking buffer (Invitrogen), sections were incubated with anti-NICD2 antibodies at 4°C overnight. Sections were extensively washed with PBS and subsequently incubated with HRP-conjugated secondary antibody for 1 hour at RT. The tyramide signal amplification (TSA) was used as per manufacturer's instructions (Invitrogen).

To quantify lumen diameter and ciliated luminal cells, entire pancreata of *Sox9$^{flox/flox}$* and *Sox9$^{flox/flox}$; Rosa26-CreER* embryos at E15.5 were sectioned at 10 μm. *z*-stack (~7 μm thick) Apotome images were taken and 3D rendered by AxioVision 4.8 software. Five 3D images per embryo and three embryos for each genotype were analyzed. The lumen diameter was measured using the 'length tool' (AxioVision 4.8) placed in the largest opening of the lumens in a given image. For quantification of ciliated cells, the total number of E-cadherin$^+$ epithelial cells contacting lumens were counted and the percentage of acetylated-α-tubulin$^+$ cells determined.

Pancreatic explants and transient transfections

Dorsal and ventral buds were dissected from mouse embryos at E12.5 and cultured in DMEM/F12 with 10% fetal bovine serum on top of microporous filters. In select experiments, mesenchyme was mechanically removed after incubating E12.5 pancreatic rudiments in 4 mg/ml collagenase B (Roche Applied Science) at 37°C for 10 minutes. Rudiments were then embedded in 50 μl 50% growth factor reduced Matrigel (BD Bioscience) and the growth medium supplied with 100 ng/μl Fgf10 and Fgf7 (R&D Systems). γ-secretase inhibitors IX and XX (GSI-IX and GSI-XX, Calbiochem) at a final concentration of 1-10 μM and 0.01-1 μM, respectively, were added to the culture medium for 24-72 hours. For *Pdx1-CreER; Rosa26-NotchIC* explants, pregnant dams received an i.p. injection of tamoxifen at E10.5, pancreata were dissected at E12.5, and cultured in 5 μM 4-hydroxy tamoxifen (Sigma) and 5 μM GSI-IX for three days. *Sox9$^{flox/flox}$* and *Rosa26-CreER; Sox9$^{flox/flox}$* explants were cultured in 5 μM 4-hydroxy tamoxifen for two days followed by one day of recovery before GSI-IX treatment.

A 630 bp mouse *Ngn3* cDNA fragment was PCR amplified and cloned into the *pCMV-IRES-eGFP* (Clontech) vector. Transfections of HepG2 cells were performed as described (Shih et al., 2007).

Fluorescent activated cell sorting, microarray analysis and mRNA quantification

Total RNA was isolated and pooled from pancreatic epithelia dissected from *Sox9$^{flox/flox}$; Rosa26-CreER* and *Sox9$^{flox/flox}$* littermates at E15.5. Each individual RNA sample was prepared from four pancreata as per the manufacturer's instructions (Micro RNA isolation kit, Qiagen). RNA quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Approximately 250 ng of total RNA was amplified and labeled with Cy3 using the QuickAmp Labeling Kit (Agilent Technologies). Four independent samples were hybridized to Agilent Whole Mouse Genome Oligo Microarray G4122A chips. Microarray data are available at Gene Expression Omnibus (GEO) under accession GSE28670.

Total RNA was isolated from sorted eGFP$^+$ and eGFP$^-$ cell fractions from two 23-day-old *Sox9-eGFP* mice. For fluorescent activated cell sorting, tissue was first dissociated with collagenase B/trypsin, and sorted on a FACSAria II (BD Biosciences). Three independent samples were hybridized to an Affymetrix Mouse Gene 1.0 ST array. Microarray data are available at GEO under accession GSE34060.

For qRT-PCR analyses, cDNA was synthesized (Superscript III cDNA kit; Invitrogen) from total RNA pooled from pancreatic epithelia from four explants. qRT-PCRs were performed in four replicates using SYBR Green (Applied Biosystems). qRT-PCR primer sequences can be found in supplementary material Table S2.

Statistical analysis

All values are shown as mean ± standard error of the mean (s.e.m.). *P*-values were calculated using Student's two-tailed *t*-test; $P<0.05$ was considered to be significant.

RESULTS

Sox9$^+$ cells in the developing pancreas are Notch responsive

To define the Notch signaling niche during the major time period of pancreatic endocrine differentiation, we performed co-immunofluorescence analysis for Notch receptors together with known Notch downstream effectors, Rbpj and Hes1, as well as Sox9 and Ngn3. Because *Notch3* and *Notch4* are predominantly expressed in mesenchymal and endothelial cells (Lammert et al., 2000), we focused our analysis on the epithelial Notch receptors Notch1 and Notch2. At E15.5, strongest Notch1 immunoreactivity was detected in acinar cells, whereas Sox9$^+$ and Hes1$^+$ cells in the epithelial cords displayed weaker expression. Ngn3$^+$ and endocrine cells were devoid of Notch1 (Fig. 1A-C). Notch2 was mostly confined to centrally located Hes1$^+$ and Sox9$^+$ cells, but was not detected in endocrine or acinar cells (Fig. 1D,E). A subset of

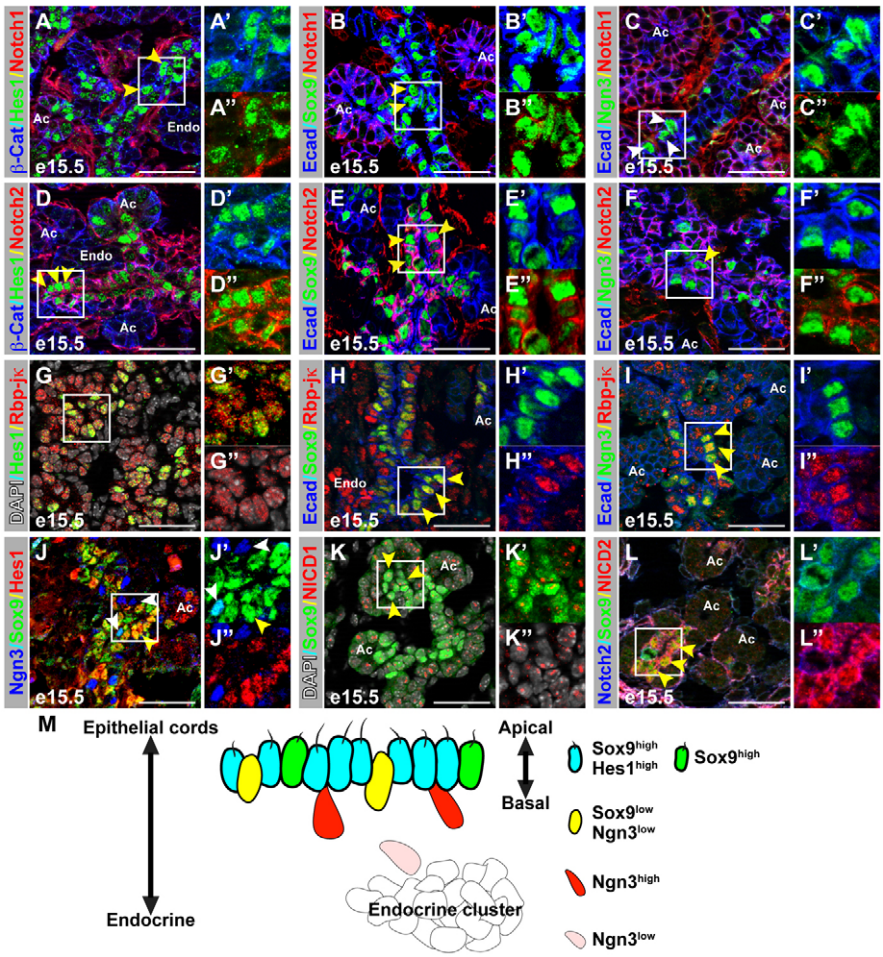

Fig. 1. Sox9⁺ epithelial cord progenitors are Notch active. (**A-L″**) Immunofluorescence staining of mouse pancreas at E15.5 shows weak Notch1, but strong Notch2, and Rbpj expression in Hes1⁺ and Sox9⁺ cells of the progenitor epithelium (yellow arrowheads in A,B,D,E,H). Ngn3⁺ cells also express Notch2 and Rbpj (yellow arrowheads in F,I) but little Notch1 (white arrowheads in C). Hes1 is expressed in a subpopulation of Sox9⁺ cells that is Ngn3⁻ (yellow arrowhead in J). Ngn3⁺ or Sox9⁺/Ngn3⁺ cells do not express Hes1 (white arrowheads in J,J′). The majority of Sox9⁺ cells express NICD1 and NICD2 (yellow arrowheads in K,L). β-Catenin (β-Cat), E-cadherin (Ecad) and DAPI visualize epithelial cells or cell nuclei. Insets show higher magnifications of boxed areas. (**M**) Graphic illustration of the Hes1, Sox9 and Ngn3 expression domains in the epithelial cords at E15.5. Ac, acinar; Endo, endocrine cluster; NICD, Notch intracellular domain. Scale bars: 40 μm.

luminal Ngn3⁺ cells retained Notch2 expression, but Notch2 immunofluorescence intensity was reduced in delaminating Ngn3⁺ cells (Fig. 1F). These findings demonstrate that epithelial cord progenitors are competent to transduce Notch signals. This notion is further supported by expression of Rbpj and Hes1 (Fig. 1G-I), as well jagged 1 and, to a lesser extent, Dll1 in this domain (supplementary material Fig. S1A-F).

To determine whether Sox9⁺ cells are Notch signaling active at E15.5, we assessed the extent of overlap between Sox9, Hes1 and Ngn3. Only a subpopulation of Sox9⁺ cells expressed Hes1, and high Ngn3 levels were only observed in Sox9⁺ but never in Hes1⁺ cells (Fig. 1J,M). To gauge more directly Notch activity in the Sox9⁺ domain, we determined whether Sox9⁺ cells exhibit nuclear expression of NICD1 and NICD2. Despite weaker expression of Notch1 than Notch2 in Sox9⁺ cells (Fig. 1B,E), nuclear NICD1 and NICD2 were detected in the majority of Sox9⁺ cells (Fig. 1K,L; supplementary material Fig. S2A-M), indicating that Sox9⁺ cells are Notch signaling active. Together with previous observations that NICD1 and Sox9 are downregulated in Ngn3⁺ cells (Magenheim et al., 2011; Seymour et al., 2008), the finding that Ngn3⁺ cells are Hes1⁻ (Fig. 1J,M) indicates that induction of Ngn3 and delamination from the epithelial cords is associated with reduced Notch activity.

Hes1 and Sox9 are Notch dependent but respond at different thresholds

Nuclear localization of NICD in Sox9⁺ cells suggests that Sox9 might be regulated by Notch, as previously observed in liver (Zong et al., 2009). Curiously, the Notch target Hes1 was only expressed in a subset of Sox9⁺ cells, suggesting that the two factors might respond to Notch signaling at different thresholds. To test this idea, we blocked Notch signaling by culturing whole pancreatic explants from *Sox9-eGFP* embryos at E12.5 in the presence of different concentrations of γ-secretase inhibitor-IX (GSI-IX) and studied the effects on Sox9 and Hes1 expression. GSIs block Notch proteolytic cleavage, thereby preventing generation of the NICD (Shih Ie and Wang, 2007). Addition of GSI-IX to the culture medium resulted in a significant reduction of Hes1 mRNA and protein at 1 μM GSI-IX (Fig. 2A,B,I), a concentration that had no effect on Sox9 expression (Fig. 2E,F,I; supplementary material Fig. S3A,B,E,F, Table S3). By contrast, the expression of Hes1 and Sox9 was both severely diminished at 5 μM or 10 μM GSI-IX (Fig. 2C,D,G-I; supplementary material Fig. S3C,D,G,H, Table S3), demonstrating that Hes1 and Sox9 are Notch target genes, but respond to Notch at different thresholds. Similar inhibition of Sox9 expression was observed with a chemically different GSI (GSI-XX) (supplementary material Fig. S3I-Q). Further confirming Notch-dependency of the GSI effects, treatment of explants with GSI-IX failed to downregulate Hes1 and Sox9, when the NICD was constitutively misexpressed in pancreatic epithelial cells (supplementary material Fig. S4A-E). Sox9 and Hes1 expression were also reduced in mesenchyme-depleted pancreatic explants treated with 5 μM GSI-IX (supplementary material Fig. S5A-D), demonstrating that Notch exerts its effects on Sox9 and Hes1 directly in epithelial cells. Together, these findings suggest that Hes1 and Sox9 are co-expressed when Notch activity is high in epithelial cells, whereas at intermediate Notch activity only Sox9,

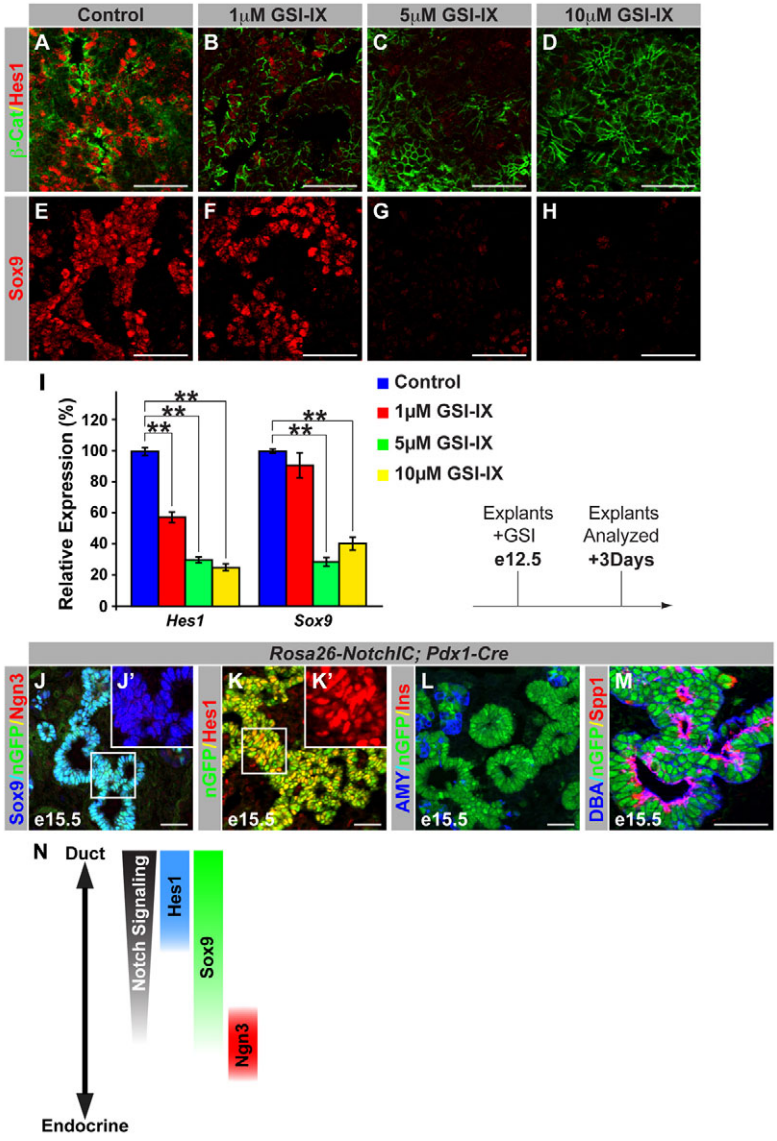

Fig. 2. Notch signaling activates Hes1 and Sox9 at different thresholds. (**A-H**) Immunofluorescence staining of pancreatic explants from E12.5 *Sox9-eGFP* embryos cultured for 3 days in the presence of three different concentrations of γ-secretase inhibitor-IX (GSI-IX). At 1 μM GSI-IX, Hes1 expression is reduced, whereas Sox9 expression is maintained (B,F). At 5 μM and 10 μM GSI-IX, both Hes1 and Sox9 are severely diminished (C,D,G,H). (**I**) Quantitative RT-PCR analysis of *Hes1* and *Sox9* mRNA in explants cultured in different concentrations of GSI-IX (n=4). (**J-M**) Immunofluorescence staining of pancreata from *Rosa26-NotchIC; Pdx1-Cre* embryos at E15.5 demonstrates uniform Sox9 expression in NICD-expressing cells (marked by nGFP) (J). All NICD-expressing cells also express elevated level of Hes1 and a subset of targeted cells exhibit a Hes1^{high} state (K). nGFP^{+} cells express the ductal marker Spp1 and DBA (M), but not Ngn3 (J), insulin (Ins) or amylase (AMY) (L). Insets show higher magnifications of boxed areas. (**N**) Graphical summary, correlating Notch activity with Hes1, Sox9 and Ngn3 expression. At high Notch activity, Hes1 and Sox9 are co-expressed, whereas intermediate Notch levels maintain Sox9, but not Hes1. β-Cat, β-catenin; DBA, *Dolichos biflorus* agglutinin. Values are shown as mean ± s.e.m. $^{**}P$<0.01. Scale bars: 40 μm.

but not Hes1, is expressed. This provides an explanation for why Sox9 is expressed in a broader domain of the epithelial cords than Hes1 and suggests that Notch does not operate in a simple on-off mode, but that gradients of Notch activity in epithelial cord progenitors produce distinct cellular states.

These findings imply that Sox9 should respond more readily to an increase in Notch activity than Hes1. To test this notion, we forced expression of the NICD in pancreatic progenitors by *Pdx1-Cre*-mediated recombination of the *Rosa26-NotchIC* allele in mice. As previously described (Murtaugh et al., 2003), NICD misexpression resulted in a tubular, multilayered epithelium, and a complete block in endocrine and acinar cell differentiation (Fig. 2J-M). Expression of osteopontin (Spp1) (Kilic et al., 2006) in these epithelial cysts (Fig. 2M) indicates that NICD-expressing cells exhibit duct-like features. Strikingly, Sox9 was expressed throughout the entire NICD^{+} epithelium (94.3±2.8% of nGFP^{+} cells expressed Sox9 in *Rosa26-NotchIC; Pdx1-Cre* pancreata, n=3; 574 cells counted) (Fig. 2J; supplementary material Fig. S4C), suggesting that high Notch activity readily induces Sox9. Notably, although NICD misexpression also induced Hes1, only a subset of targeted cells exhibited a Hes1^{high} state, whereas a considerable number of NICD^{+}/Sox9^{+} cells displayed intermediate levels of Hes1 (31.2±7.4% of nGFP^{+} cells expressed Hes1^{high} and 46.4±6.0% of nGFP^{+} cells expressed Hes1^{intermediate} in *Rosa26-NotchIC; Pdx1-Cre* pancreata, n=3; 663 cells counted) (Fig. 2K; supplementary material Fig. S4C). These observations further support the notion that Hes1 expression requires higher Notch activity than does Sox9 (Fig. 2N).

Sox9 is a cell-autonomous positive regulator of endocrine development

Our own studies have shown that *Sox9* haploinsufficiency causes reduced numbers of endocrine cells (Seymour et al., 2008), implying that *Sox9* is required for endocrine development. Its regulation by Notch, however, appears to be inconsistent with a pro-endocrine function of Sox9, as Notch signaling has a well-established role in blocking endocrine differentiation (Apelqvist et al., 1999; Magenheim et al., 2011; Murtaugh et al., 2003; Nakhai et al., 2008). To begin to resolve this apparent conundrum, we examined the role of Sox9 in endocrine differentiation specifically during the secondary transition. To this end, we generated *Sox9$^{flox/flox}$; Rosa26-CreER* embryos and deleted *Sox9* by administering tamoxifen i.p. to pregnant dams at E12.5 (hereafter

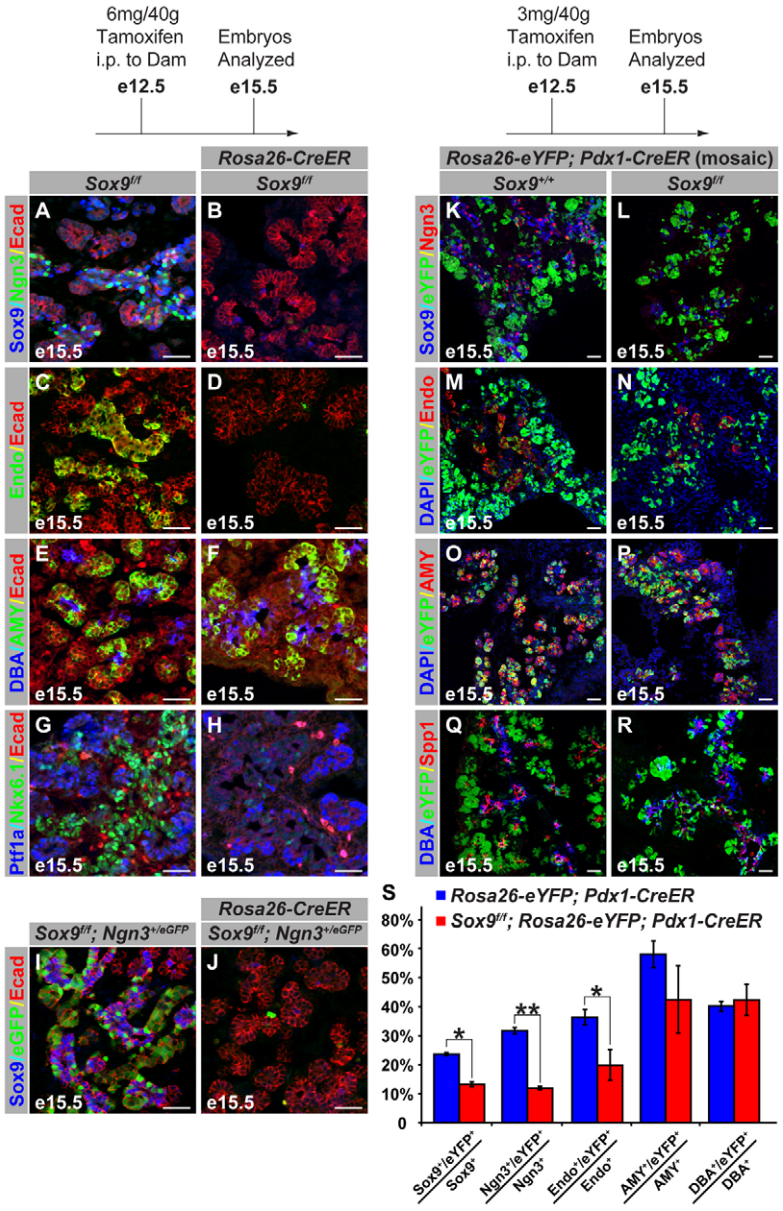

Fig. 3. *Sox9* cell-autonomously controls endocrine differentiation. (**A-J**) Immunofluorescence staining of E15.5 Sox9$^{f/f}$ (A,C,E,G), Sox9$^{f/f}$; *Rosa26-CreER* (B,D,F,H), Ngn3$^{+/eGFP}$; Sox9$^{f/f}$ (I) and Ngn3$^{+/eGFP}$; Sox9$^{f/f}$; *Rosa26-CreER* (J) embryos injected with high dosage of tamoxifen at E12.5. After *Sox9* deletion, expression of Sox9 (B), Ngn3 protein (B) and *Ngn3* mRNA (assessed by eGFP in J), endocrine hormones (D) and Nkx6.1 (H) are almost completely abolished. The acinar markers amylase (AMY) (F) and Ptf1a (H), or the ductal marker DBA (F) are not affected by *Sox9* inactivation. E-cadherin (Ecad) visualizes epithelial cells. (**K-R**) Immunofluorescence staining of E15.5 *Rosa26-eYFP*; Sox9$^{+/+}$; Pdx1-CreER (K,M,O,Q) and *Rosa26-eYFP*; Sox9$^{f/f}$; Pdx1-CreER (L,N,P,R) embryos injected with low dosage of tamoxifen for mosaic *Sox9* deletion. Recombined cells are traced by eYFP. (**S**) Quantification of marker^{+}/eYFP^{+} cells relative to the total number of marker^{+} cells (n=3). *Sox9*-deficient cells show reduced propensity to differentiate into endocrine cells. Endo, all four endocrine hormones; DBA, *Dolichos biflorus* agglutinin. Values are shown as mean ± s.e.m. *P<0.05, **P<0.01. Scale bars: 40 μm.

referred to as *Sox9*$^{\Delta e13/\Delta e13}$ embryos). This regimen resulted in >90% loss of Sox9 throughout the pancreatic epithelium at E15.5 and, unlike earlier deletion (Seymour et al., 2007), affected overall organ size only mildly (Fig. 3A,B; data not shown).

In control embryos, Sox9^{+} cells form an E-cadherin^{+}, monolayered epithelium at E15.5, in which abundant Ngn3^{+} cells both inside and outside the epithelium indicate ongoing delamination of endocrine progenitors (Fig. 3A). Ngn3^{+} cells were almost completely absent from *Sox9*$^{\Delta e13/\Delta e13}$ embryos (Fig. 3B). Furthermore, as evidenced by reduced eGFP expression from the *Ngn3*^{eGFP} allele in *Sox9*$^{\Delta e13/\Delta e13}$ embryos (Fig. 3I,J), loss of *Sox9* affected *Ngn3* expression at the transcriptional level. Consistent with the severe reduction of *Ngn3* expression, *Sox9*$^{\Delta e13/\Delta e13}$ embryos displayed an almost complete absence of endocrine cells, whereas the acinar compartment was unaffected (Fig. 3C-F). Hes1 expression was maintained in the absence of Sox9, although the intensity of the immunofluorescence signal was reduced (supplementary material Fig. S6A-C,E). Together, these findings demonstrate an absolute requirement for *Sox9* in endocrine development during the secondary transition. In this process, Sox9 appears to function upstream of Nkx6.1 (Fig. 3G,H), which is a crucial Notch-induced regulator of endocrine cell specification (Schaffer et al., 2010).

The finding that Sox9 occupies *Ngn3* regulatory sequences (Lynn et al., 2007; Seymour et al., 2008) hints at a possible cell-autonomous role for Sox9 in *Ngn3* induction and endocrine cell formation. To test this hypothesis, we ablated *Sox9* mosaically in *Sox9*$^{flox/flox}$; *Rosa26-eYFP; Pdx1-CreER* embryos and traced recombined cells based on eYFP expression (Fig. 3K-R). *Sox9*-deficient (eYFP^{+}) cells exhibited a significantly reduced propensity to give rise to Ngn3^{+} and hormone-expressing cells, whereas *Sox9* deletion did not affect the ability of progenitors to differentiate into amylase^{+} acinar or DBA^{+} ductal cells (Fig. 3S). Notably, not all cells that recombined the *Rosa26-eYFP* allele also recombined the *Sox9*^{flox} allele (Fig. 3L,S), showing that the two recombination events are not entirely interdependent. However, combined with previous in vitro evidence showing that Sox9 directly stimulates *Ngn3* promoter activity (Lynn et al., 2007), our data support a cell-autonomous requirement for Sox9 in *Ngn3* induction.

Notch inhibition induces *Ngn3* expression rapidly within 24 hours (supplementary material Fig. S6D; data not shown) (Duvillie et al., 2006; Magenheim et al., 2011), resulting in increased numbers of endocrine cells 3 days after initiation of GSI treatment (supplementary material Fig. S7B-D). To determine whether Ngn3 induction requires *Sox9* when Notch signaling is reduced, we treated pancreatic explants from *Sox9*$^{flox/flox}$*; Rosa26-eYFP; Rosa26-CreER* embryos with tamoxifen to delete *Sox9* and subsequently added 5 μM GSI-IX to the culture medium to inhibit Notch signaling. As previously shown (Duvillie et al., 2006; Magenheim et al., 2011), one day of GSI treatment resulted in induction of Ngn3 in control explants (supplementary material Fig. S6C,D,G). By contrast, Notch inhibition failed to induce Ngn3 in *Sox9*-deficient explants (supplementary material Fig. S6E-G), identifying Sox9 as an obligatory inducer of *Ngn3*. These findings argue that the prevailing model whereby Notch solely inhibits differentiation needs to be refined. Instead, our data suggest that Notch induces both activators and inhibitors of endocrine differentiation and that the undifferentiated state is defined by transcriptional competition between these opposing activities (Fig. 2N).

Ngn3 provides negative feedback on Sox9 and represses the ductal program

Our finding that Sox9 is required for Ngn3 expression raises the question of why only Ngn3^{low} cells express Sox9, whereas Ngn3^{high} cells are almost uniformly Sox9^{-} (Seymour et al., 2008). To explore whether high levels of Ngn3 repress Sox9, we employed an inducible transgenic model of *Ngn3* expression to express Ngn3 uniformly in pancreatic progenitors. Induction of the *Pdx1-Ngn3ER-IRES-nGFP* transgene (Johansson et al., 2007) by tamoxifen administration at E8.75 resulted in rapid and complete repression of Sox9 throughout the pancreatic epithelium (Fig. 4A,B). A similar effect was seen when Ngn3 misexpression was induced at E11.75 (Fig. 4C), suggesting that Ngn3 functions as a potent repressor of Sox9 during the primary and secondary transition. This negative-feedback loop appears to operate cell autonomously, as mosaic misexpression of *Ngn3* in Sox9^{+} HepG2 cells also repressed Sox9 in transfected cells (Fig. 4D-F).

To exclude the possibility that this negative feedback of Ngn3 on Sox9 accounts for Sox9 downregulation in the presence of the Notch inhibitor GSI-IX, we treated pancreatic explants from *Ngn3*-deficient embryos with GSI-IX and examined Sox9 expression. High dosages of GSI-IX abolished Sox9 expression in both *Ngn3*-deficient and control explants (supplementary material Fig. S8A-D), showing that Notch exhibits direct effects on Sox9 and that Ngn3-mediated repression does not account for the loss of Sox9 in GSI-IX-treated explants. Thus, Notch first induces Ngn3 expression through Sox9, and high levels of Ngn3 in turn repress Sox9.

To define how the Ngn3^{+} domain separates from the Sox9^{+} domain, we analyzed *Ngn3*$^{+/eGFP}$ and *Ngn3*$^{eGFP/eGFP}$ embryos, in which cells exhibiting *Ngn3* promoter activity can be visualized by eGFP in the Ngn3^{+} and Ngn3-deficient state, respectively. Because Ngn3 has been shown to repress its own promoter (Smith et al., 2004), we expected to see a higher intensity of eGFP expression in *Ngn3*$^{eGFP/eGFP}$ than in *Ngn3*$^{+/eGFP}$ embryos. Contrary to this prediction, *Ngn3*-deficient embryos displayed reduced eGFP signal compared with *Ngn3*$^{+/eGFP}$ embryos (Fig. 5A-D), suggesting that *Ngn3* promotes its own expression in vivo. This positive Ngn3 auto-feedback appears to be independent of Notch activity, as GSI-IX-treated pancreatic explants from *Ngn3*$^{eGFP/eGFP}$ embryos also displayed markedly lower eGFP expression than did explants from *Ngn3*$^{+/eGFP}$ embryos (supplementary material Fig. S8B,D,F,H). These findings suggest that once Sox9 has initiated *Ngn3* expression, positive Ngn3 auto-regulation helps progenitors attain the Ngn3^{high} state, which marks commitment to the endocrine program.

Next, we sought to characterize the lineage identity of *Ngn3*-expressing cells in the absence of Ngn3 protein. In control *Ngn3*$^{+/eGFP}$ embryos, eGFP^{+} cells were largely devoid of Sox9, Hes1, Tcf2, Prox1 and Spp1 (Fig. 5E,G,I,M,O), which are markers

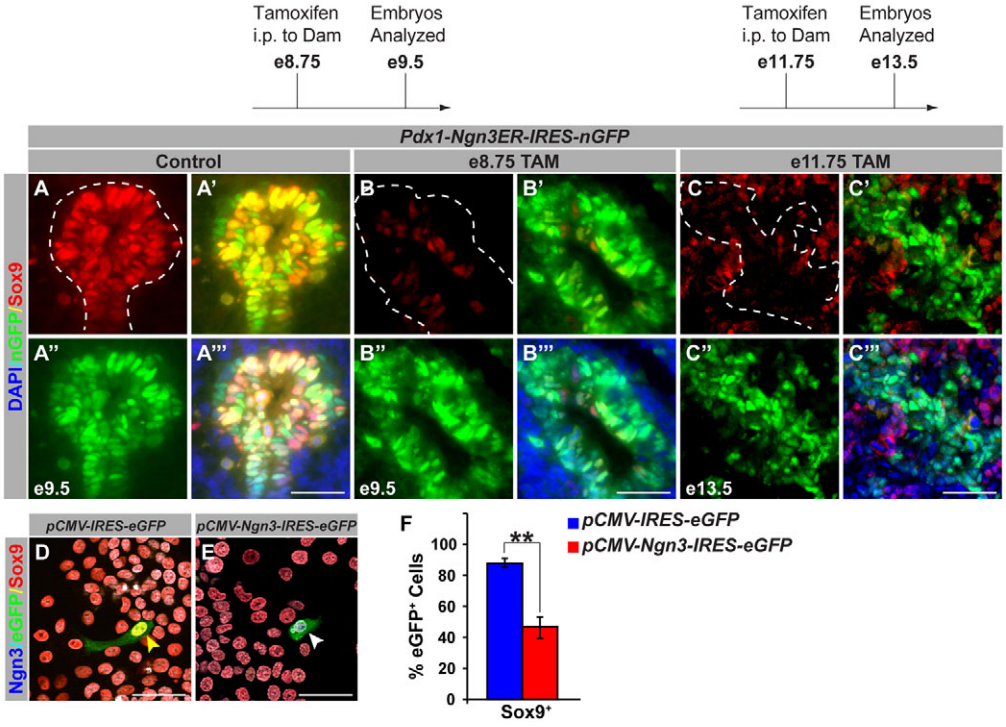

Fig. 4. Ngn3 cell-autonomously represses Sox9.
(**A-C‴**) Immunofluorescence staining of *Pdx1-Ngn3ER-IRES-nGFP* embryos injected with tamoxifen at E8.75 (B-B‴) or E11.75 (C-C‴) and analyzed at E9.5 or E13.5, respectively. Tamoxifen-mediated nuclear translocation of the Ngn3-ER^{TM} protein results in rapid and significant reduction of Sox9 (B-C‴). Dashed line outlines the pancreatic epithelium.
(**D-F**) Transient transfection of HepG2 cells reveals robust Sox9 expression in *pCMV-IRES-eGFP*- (yellow arrowhead in D), but not in *pCMV-Ngn3-IRES-eGFP*-transfected cells (white arrowhead in E).
(F) Quantification of eGFP^{+}/Sox9^{+} cells relative to the total number of eGFP^{+} cells (n=3). Values are shown as mean ± s.e.m.
**P<0.01. Scale bars: 40 μm.

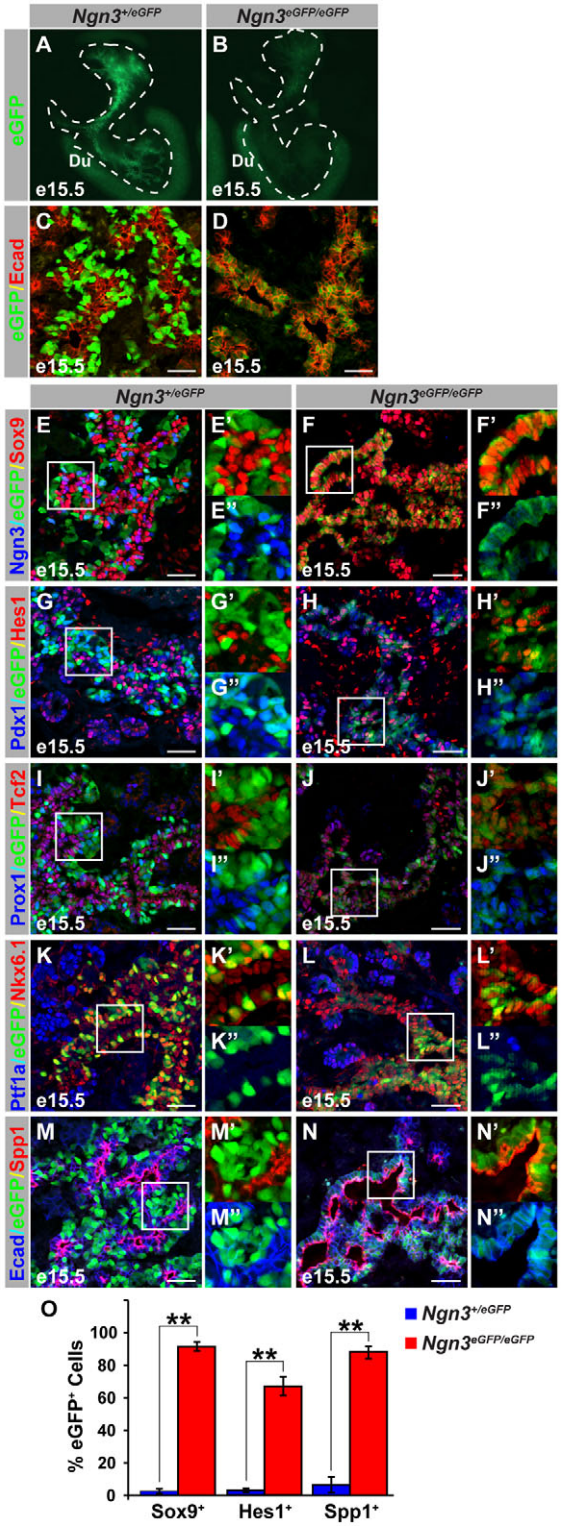

Fig. 5. *Ngn3* deficiency results in reduced *Ngn3* promoter activity and ectopic expression of ductal markers. (**A-D**) Whole-mount fluorescence image of *Ngn3*$^{eGFP/eGFP}$ and *Ngn3*$^{+/eGFP}$ embryos shows reduced eGFP signal in *Ngn3*-deficient embryos at E15.5. The pancreas is outlined by a dashed line (A,B). In *Ngn3*-deficient embryos, GFP$^+$ cells retain E-cadherin (Ecad) expression (D), suggesting failure to delaminate from the epithelial cords. (**E-N″**) Immunofluorescence staining for progenitor and ductal cell markers on pancreatic sections from *Ngn3*$^{+/eGFP}$ (E,G,I,K,M) and *Ngn3*$^{eGFP/eGFP}$ (F,H,J,L,N) embryos at E15.5. eGFP$^+$ cells in *Ngn3*$^{+/eGFP}$ pancreata are largely devoid of Sox9, Hes1, Tcf2, Prox1 and Spp1 (E,G,I,M), whereas eGFP$^+$ cells in *Ngn3*$^{eGFP/eGFP}$ embryos retain expression of these markers (F,H,J,N). The pre-acinar marker Ptf1a is not detected in eGFP$^+$ cells of either *Ngn3*$^{+/GFP}$ or *Ngn3*$^{eGFP/eGFP}$ embryos (K,L). Insets show higher magnifications of boxed areas. (**O**) Quantification of marker$^+$/eGFP$^+$ cells relative to the total number of eGFP$^+$ cells (n=3). Du, duodenum. Values are shown as mean ± s.e.m. $^{**}P<0.01$. Scale bars: 40 μm.

of bipotential ductal/endocrine progenitors in the embryo and later become restricted to the pancreatic ducts (Kopinke et al., 2011; Poll et al., 2006; Seymour et al., 2007; Wang et al., 2005; Zhang et al., 2009). In the absence of Ngn3, eGFP$^+$ cells retained Sox9, Hes1, Tcf2 (Hnf1b – Mouse Genome Informatics), Prox1 and Spp1 (Fig. 5F,H,J,N,O), demonstrating a general failure of *Ngn3*-deficient progenitors to downregulate ductal genes. Overall Hes1 immunofluorescence intensity outside the GFP$^+$ domain appeared to be lower in *Ngn3*-deficient embryos (Fig. 5G,H), which confirms previous observations and suggests a non-cell-autonomous role for Ngn3 in promoting Hes1 expression (Magenheim et al., 2011). However, our data also reveal a novel cell-autonomous role for Ngn3 in repressing Hes1 in differentiating endocrine cells. *Ngn3*-deficient progenitors did not express the pre-acinar marker Ptf1a (Fig. 5K,L), which is consistent with the finding that endocrine progenitors do not adopt acinar identity when *Ngn3* is inactivated during the secondary transition (Beucher et al., 2011). Together, our data suggest that Ngn3 represses ductal programs and that in the absence of Ngn3 activity, bipotential ductal/endocrine progenitors adopt a ductal fate.

Sox9 is required for ductal cell differentiation and maintenance

Given the exclusive expression of Sox9 in pancreatic ductal cells of the postnatal pancreas, we wondered whether Sox9 could play a role in ductal cell differentiation. To address this question, we analyzed *Sox9*$^{\Delta e13/\Delta e13}$ embryos for possible ductal defects. *Sox9*$^{\Delta e13/\Delta e13}$ embryos exhibited a disorganized ductal network with dilated lumens (Fig. 6B,C,J). When *Sox9* was inactivated at E11 or when *Sox9*$^{\Delta e13/\Delta e13}$ embryos were analyzed postnatally, we observed a fully penetrant pancreatic cystic phenotype (supplementary material Fig. S9A-G). Similar to embryonic inactivation, conditional *Sox9* deletion in adult mice also resulted in a polycystic pancreas (supplementary material Fig. S9A,H-K), demonstrating a requirement for *Sox9* in establishing and maintaining the ductal epithelium. Notably, the development of cysts upon adult *Sox9* inactivation demonstrates that the cystic phenotype is not a consequence of blocked endocrine differentiation, but reflects a direct requirement for *Sox9* in pancreatic ductal cells.

To gain insight into how Sox9 controls ductal cell differentiation, we compared the transcriptional profiles of pancreata from *Sox9*$^{\Delta e13/\Delta e13}$ mice with those of control littermates. We then selected for Sox9-regulated genes with a >1.5-fold change and FDR<0.05 that were specifically enriched in Sox9$^+$ ductal cells of the adult pancreas. Employing these criteria, we identified 84 Sox9-regulated ductal genes of which 15 showed a >2-fold decrease in *Sox9*-deficient pancreata (Fig. 6A; supplementary material Table S4). Among these genes was

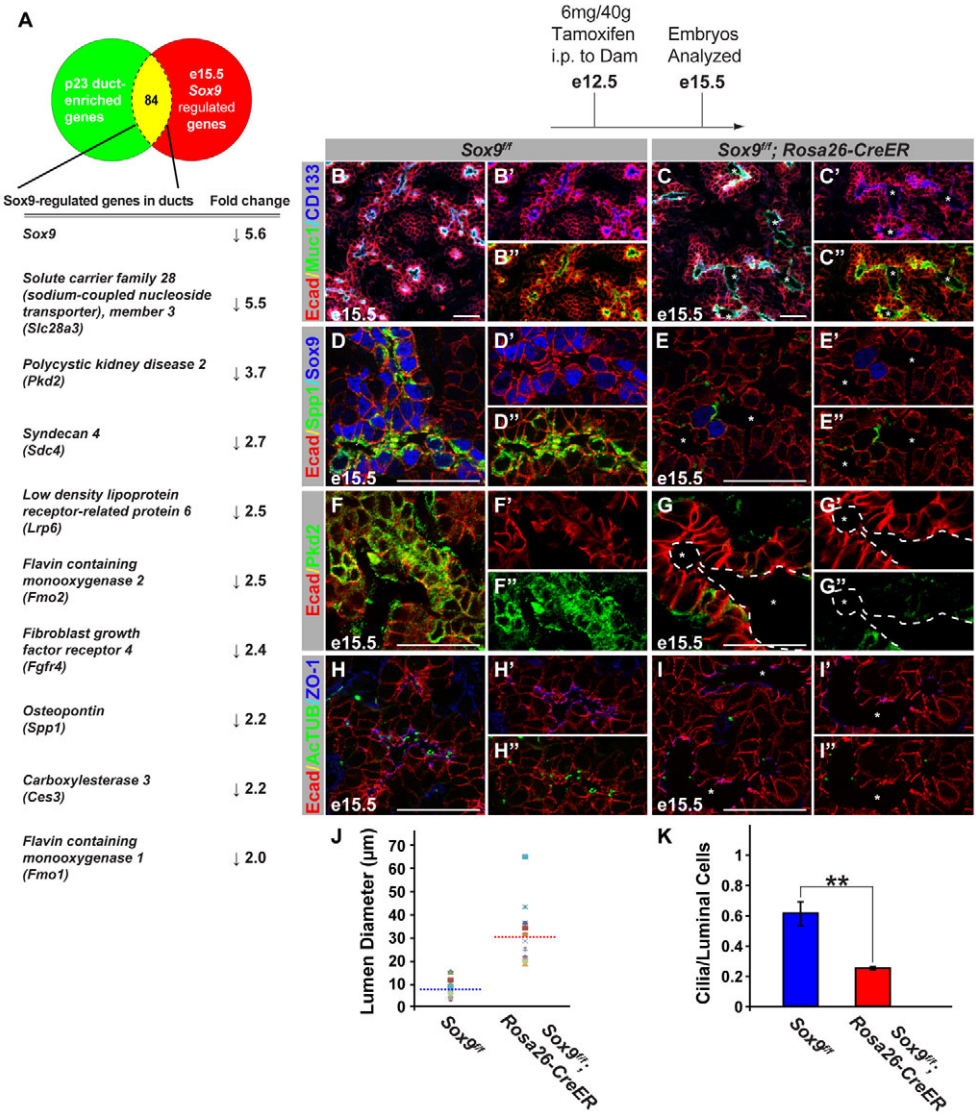

Fig. 6. *Sox9* is required for establishing a ductal epithelium. (**A**) *Sox9*-regulated genes were identified by comparing mRNA profiles of E15.5 pancreata from *Sox9*$^{f/f}$*; Rosa26-CreER* and *Sox9*$^{f/f}$ littermates after inducing *Sox9*-deletion by tamoxifen injection at E12.5 (>1.5-fold change; *n*=4) and duct-enriched genes by comparing eGFP^{+} and eGFP^{-} populations in *Sox9*-eGFP mice at p23 (*n*=3). The ten genes exhibiting a >2-fold decrease in *Sox9*-deficient embryos are listed. (**B-I″**) Immunofluorescence staining for ductal cell markers on pancreatic sections of Sox9$^{f/f}$ (B,D,F,H) and *Sox9*$^{f/f}$*; Rosa26-CreER* (C,E,G,I) embryos injected with tamoxifen at E12.5 and analyzed at E15.5. *Sox9* deficiency results in dilated lumens (asterisks in C,E,G,I). Expression of apical markers mucin 1 (Muc1), prominin 1 (CD133) (C) and ZO-1 (I) are not affected by *Sox9* deletion. However, *Sox9* inactivation results in loss of apically secreted Spp1 (E), polycystic kidney disease 2 (Pkd2; G) and the primary cilia marker acetylated-α-tubulin (AcTUB; I). E-cadherin (Ecad) visualizes epithelial cells. (**J**,**K**) Quantification of lumen diameter and ciliated luminal cells at E15.5 (*n*=3). Values are shown as mean ± s.e.m. ***P*<0.01. Scale bars: 40 μm.

the known ductal marker Spp1 (Kilic et al., 2006) (Fig. 6A,D,E; supplementary material Fig. S9L,M), which was identified as a direct Sox9 target gene in chondrocytes (Peacock et al., 2011). In addition, our analysis unveiled novel putative ductal markers, such as *Sdc4*, *Lrp6*, *Fgfr4* and polycystin 2 (*Pkd2*). Especially intriguing was the discovery of *Pkd2* as a Sox9-regulated ductal marker, as *Pkd2* mutations are associated with autosomal dominant polycystic kidney disease (ADPKD), a disorder characterized by the formation of cysts in kidney, liver and pancreas (Wu et al., 1998; Wu et al., 2000). Immunofluorescence staining for Pkd2 revealed robust membranous expression specifically in the epithelial cords (Fig. 6F). In *Sox9*$^{\Delta e13/\Delta e13}$ embryos, epithelial cords were largely devoid of Pkd2 (Fig. 6G), demonstrating a requirement for *Sox9* in maintaining Pkd2 expression in developing ducts.

Given the known role of Pkd2 in maintaining primary cilia (Kim et al., 2009), we sought to determine whether *Sox9* deletion affects ciliogenesis in the pancreas. Immunofluorescence staining for the cilial marker acetylated-α-tubulin (AcTUB) revealed primary cilia on the apical surface of epithelial cells facing embryonic and adult ductal lumens (Fig. 6H; supplementary material Fig. S9N). Strikingly, AcTUB signal was markedly reduced in *Sox9*$^{\Delta e13/\Delta e13}$ embryos as well as in the pancreas of adult *Sox9*-deficient mice (Fig. 6I,K; supplementary material Fig. S9O), showing that *Sox9* is required for primary cilia formation. Importantly, the defect in cilia formation did not seem to be attributable to perturbed apical/basal polarity, as judged by apical localization of mucin 1, prominin (CD133) and ZO-1 (Tjp1 – Mouse Genome Informatics) in *Sox9*-deficient pancreatic epithelial cells (Fig. 6B,C,H,I). Thus, maintenance of primary cilia appears to be directly controlled by Sox9.

Sox9 is an obligatory ductal cell fate determinant downstream of Notch

Consistent with the notion that Sox9 is required for ductal cell differentiation, absence of Sox9 due to Notch inhibition was associated with loss of the ductal marker Spp1 (supplementary material Fig. S7C,D, Fig. S8F,H). To examine more rigorously the genetic interaction of Notch and Sox9 in ductal cell fate determination, we utilized *Ngn3-Cre* or *Pdx1-Cre* transgenes to recombine the *Rosa26-NotchIC* allele and force expression of NICD. We then compared the effects of NICD overexpression on ductal and endocrine gene expression in Sox9^{+} and *Sox9*-deficient states. As expected, cells targeted by the *Ngn3-Cre* transgene were

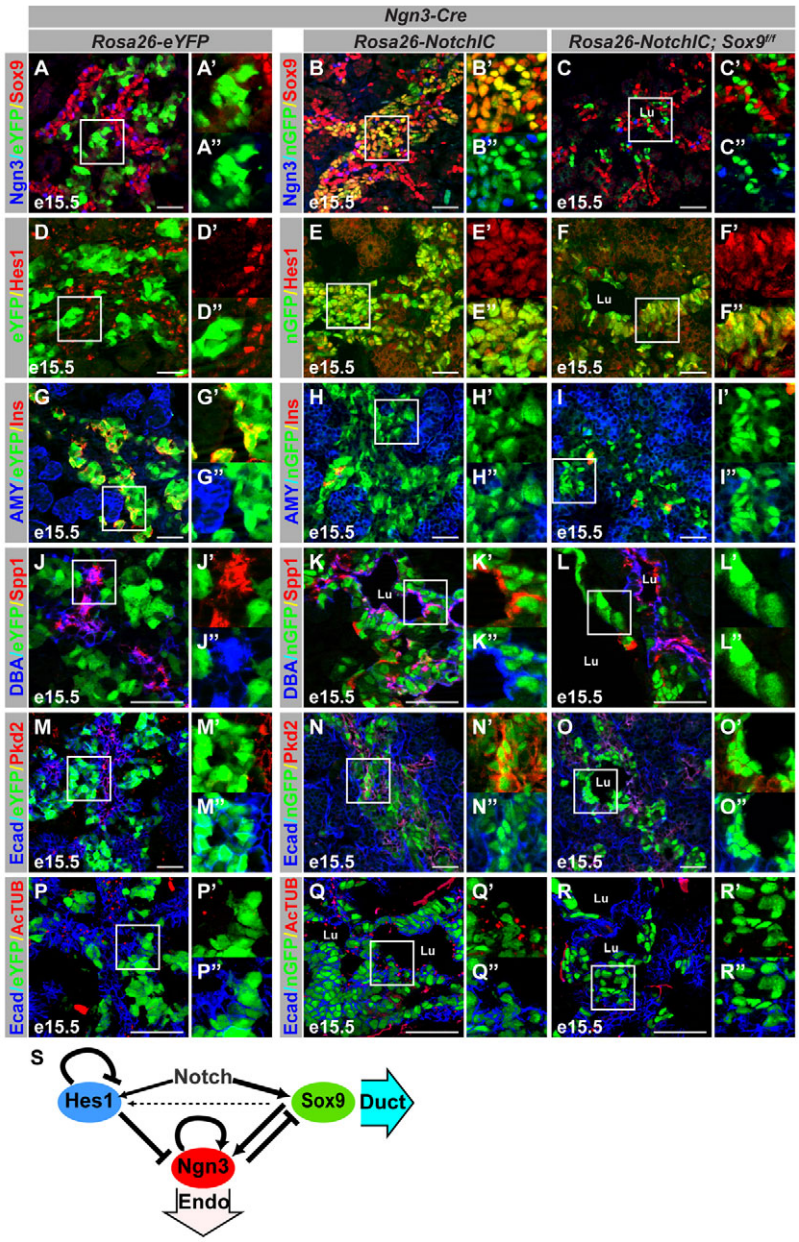

Fig. 7. High Notch activity in the absence of Sox9 does not restore endocrine or ductal cell differentiation. (**A-R″**) Immunofluorescence staining for ductal, endocrine and acinar markers on E15.5 pancreatic sections from *Rosa26-eYFP; Ngn3-Cre* (A,D,G,J,M,P), *Rosa26-NotchIC; Ngn3-Cre* (B,E,H,K,N,Q) and *Rosa26-NotchIC; Sox9$^{f/f}$; Ngn3-Cre* (C,F,I,L,O,R) embryos. In *Rosa26-eYFP; Ngn3-Cre* embryos, the majority of targeted cells, traced by eYFP, express insulin (Ins), but not ductal or acinar markers. Activation of NICD in *Ngn3*-expressing cells forces cells to express ductal markers and inhibits endocrine or acinar differentiation. Deletion of *Sox9* in NICD^{+} cells results in loss of ductal markers, Spp1, Pkd2, DBA, acetylated-α-tubulin (AcTUB) (L,O,R) and fails to restore endocrine differentiation (C,I). Insets show higher magnifications of boxed areas and E-cadherin (Ecad) visualizes epithelial cells. (**S**) Our data support a model whereby Notch signaling induces inhibitors and activators of endocrine differentiation at different thresholds. At high Notch activity Hes1-mediated *Ngn3* repression prevents Sox9 from inducing *Ngn3*. At intermediate Notch levels, Hes1 is not maintained, allowing Sox9 to enable a self-promoting molecular circuitry that drives endocrine differentiation. Negative feedback of Ngn3 on Sox9 ensures exit from the bipotential ductal/endocrine state and terminal endocrine commitment. Cells remaining Sox9^{+}/Hes1^{+} will acquire a ductal fate. Lu, lumen; DBA, *Dolichos biflorus* agglutinin. Scale bars: 40 μm.

devoid of Sox9 and Hes1 in control embryos not carrying the *Rosa26-NotchIC* allele (Fig. 7A,D). By contrast, forced NICD expression induced Sox9 in virtually all targeted cells (Fig. 7B) and, similarly, Hes1 in a large subset of cells (Fig. 7E). NICD-expressing cells also activated Sox9-regulated ductal markers, such as Spp1, Pkd2 and AcTUB, which were absent from the targeted cell population in control embryos (Fig. 7J,K,M,N,P,Q). Consistent with previous studies of NICD expression in *Pdx1*-, *Pax4*- and *Ngn3*-expressing cells (Greenwood et al., 2007; Murtaugh et al., 2003), Notch activation in endocrine progenitors severely impaired endocrine differentiation (Fig. 7G,H). Notably, some endocrine cells still arose, probably reflecting a gradual loss in competency of cells to revert to a ductal fate after *Ngn3* has been activated.

To define further the role of Sox9 under conditions of constitutively high Notch activity, we inactivated *Sox9* in *Rosa26-NotchIC; Ngn3-Cre* and *Rosa26-NotchIC; Pdx1-Cre* mice. Most *Sox9*-deficient, NICD-expressing cells maintained Hes1 expression (Fig. 7F; supplementary material Fig. S10D), confirming that Sox9 functions as a modulator rather than as an obligatory activator of Hes1 (supplementary material Fig. S6A,B). In accordance with *Sox9* being necessary for endocrine cell differentiation, endocrine cells failed to form when *Sox9* was deleted in the context of Notch activation (Fig. 7I; supplementary material Fig. S10C). Importantly, in the absence of Sox9 activity NICD misexpression was not sufficient to bestow ductal characteristics upon progenitors, as evidenced by the lack of Spp1, Pkd2 and AcTUB in targeted cells (Fig. 7L,O,R; supplementary material Fig. S10E). Thus, Sox9 is an obligatory effector of Notch-induced ductal cell differentiation.

Together, our data reveal a previously unknown Notch-dependent molecular network that balances the emergence of endocrine and ductal cells from a bipotential progenitor domain (Fig. 7S). By inducing the *Ngn3* activator Sox9, Notch renders pancreatic progenitors competent to undergo endocrine differentiation. Intermediate levels of Notch activity promote *Ngn3* induction by favoring Sox9 over Hes1 expression, demonstrating that Notch functions in a dosage-dependent manner and that

different Notch signaling levels produce distinct progenitor populations. Once Ngn3 auto-positive feedback moves progenitors into a Ngn3^{high} state, negative feedback of Ngn3 on Sox9 ensures Sox9 downregulation, which is required for repression of ductal programs and subsequent endocrine differentiation. Cells remaining Sox9$^+$/Hes1$^+$ will default into a ductal fate. Cumulatively, our findings suggest that high levels of Notch simultaneously specify endocrine and ductal lineages and that modulation of Notch activity triggers a self-perpetuating molecular circuitry that drives endocrine differentiation.

DISCUSSION

Several recent studies are in apparent discordance with the notion that Notch is a mere inhibitor of cell differentiation. For example, *Dll1*-, *Jag1*- or *Rbpj*-deficient pancreata display, at least transiently, reduced numbers of Ngn3$^+$ cells (Ahnfelt-Ronne et al., 2011; Fujikura et al., 2006; Golson et al., 2009; Nakhai et al., 2008), suggesting a possible pro-endocrine role for Notch. By demonstrating that Notch induces expression of the *Ngn3* gene activator Sox9, our study provides further evidence that Notch specifies the endocrine lineage. Our results support a model whereby under Notch^{high} conditions, the repressive effect of Hes1 on *Ngn3* overrides the activating function of Sox9 on *Ngn3*, whereas at intermediate Notch activity, reduced levels or absence of Hes1 allow Sox9 to induce *Ngn3* expression and trigger endocrine differentiation. Consistent with this notion, we observed Hes1 expression in only a subset of Sox9$^+$ cells and found Sox9$^+$/Ngn3$^+$ cells to be devoid of Hes1. Our model is also supported by recent lineage-tracing studies showing that Hes1$^+$ cells rarely undergo endocrine differentiation after E13.5 (Kopinke et al., 2011), whereas Sox9$^+$ cells give rise to endocrine cells until birth (Kopp et al., 2011).

These findings raise the question of how Notch signaling can induce Hes1 and Sox9 at different thresholds. One possible mechanism that could account for Sox9, but not Hes1, induction at intermediate Notch activity is that Sox9 reinforces its own expression, whereas Hes1 does not (Hirata et al., 2002; Kageyama et al., 2008; Lynn et al., 2007; Shimojo et al., 2008). Thus, when Notch activity decreases, Sox9 expression can be maintained owing to positive auto-regulation, whereas Hes1 levels rapidly decline.

Additional insight into how endocrine differentiation is transcriptionally regulated emerges from our discovery that Ngn3 enforces its own expression. This contrasts with prior in vitro findings that Ngn3 represses its own promoter (Smith et al., 2004). Our findings suggest that negative auto-feedback of Ngn3 on its own promoter plays, if any, only a minor role in vivo. The decrease in *Ngn3* promoter activity seen in *Ngn3*-deficient mice might not reflect auto-regulation of Ngn3, but instead might be due to loss of Ngn3-dependent positive regulators of *Ngn3* expression. One possible candidate is Myt1, which has been shown to form a feed-forward loop with Ngn3 (Wang et al., 2008).

The discovery that high levels of Ngn3 exert cell-autonomous negative feedback on Sox9 raises the question of whether endocrine differentiation requires Sox9 downregulation. Several observations are in favor of this notion. First, differentiated endocrine cells retain virtually no Sox9 expression (Seymour et al., 2007). Second, our studies show that NICD-induced *Sox9* misexpression in *Ngn3*-expressing cells prevents their endocrine differentiation and forces Ngn3$^+$ progenitor cells to adopt ductal features. Finally, analogous to our findings in pancreas, neuronal and chondrocyte precursors require Sox9 activity for their differentiation, but also need to downregulate Sox9 to complete the differentiation process (Akiyama et al., 2002; Scott et al., 2010), hinting at a conserved mechanism through which Sox9 controls cell differentiation.

Surprisingly, we found that a cell-autonomous mechanism underlies both the induction of *Ngn3* by Sox9 and subsequent negative-feedback inhibition of Sox9 by Ngn3. At first glance, this cell-autonomous mechanism appears to contradict established functions of Notch, whereby high levels of Dll1 in differentiating cells act via lateral inhibition to downregulate Ngn3 in neighboring cells (Apelqvist et al., 1999; Jensen, 2004; Jensen et al., 2000). However, the two mechanisms are not mutually exclusive and it is possible that Notch enables both cell-autonomous and non-cell-autonomous feedback mechanisms that function synergistically in endocrine differentiation. Cumulative evidence from our and recent studies favors this notion. Although examination of Notch components in *Ngn3*-deficient embryos supports the view that Ngn3$^+$ cells provide signals to undifferentiated cells through lateral inhibition (Magenheim et al., 2011), recent analysis of *Hes1* and *Dll1* mutants also revealed findings inconsistent with the lateral inhibition model and has led to the proposal that Dll1 cell-autonomously regulates Notch signaling (Ahnfelt-Ronne et al., 2011). More rigorous examination of how progenitors integrate these different signals will require a simpler assay system in which transcriptional outputs can be readily quantified at a single-cell level.

It has been proposed that Notch signaling is not required for pancreatic duct formation, but is required to promote duct differentiation passively by preventing differentiation of other cell types (Greenwood et al., 2007). Contrary to this model, our findings suggest that through regulation of *Sox9*, Notch is required to establish and maintain the ductal epithelium. Consistent with established roles of Pkd2 in ductal epithelial cells (Wu et al., 1998; Wu et al., 2000), reduced Pkd2 expression in *Sox9*-deficient ducts was associated with the formation of pancreatic cysts devoid of primary cilia. Because this phenotype was also caused by *Sox9* ablation in adult ducts, our data suggest a direct role of Sox9 in controlling epithelial properties and argue against cysts being a mere consequence of improper ductal morphogenesis caused by the lack of endocrine progenitor cell delamination due to *Ngn3* deficiency (Gouzi et al., 2011; Magenheim et al., 2011).

A genetic and functional link between Notch and ciliogenesis has been recognized previously in other epithelial organs, including skin and lung (Ezratty et al., 2011; Tsao et al., 2009). In the pancreas, Sox9 might not be the only Notch target involved in ciliogenesis. Similar to Sox9, Tcf2 is regulated by Notch (Zong et al., 2009) and loss of Tcf2 activity causes a cystic phenotype and reduced expression of the cystic disease genes *Pkhd1*, *Cys1* and *Pkd1* in kidney (Gresh et al., 2004). It is likely that Tcf2 controls the same genes in pancreas, as loss of the *Tcf2* upstream regulator Onecut1 (Hnf6) results in the formation of pancreatic cysts, loss of primary cilia, and reduced *Pkhd1*, *Cys1* and *Pkd1* expression (Pierreux et al., 2006; Zhang et al., 2009). Given that Sox9 controls *Pkd2*, but not *Pkhd1*, *Cys1* or *Pkd1*, our findings suggest the existence of multiple independent mechanisms for ciliogenesis in pancreatic ducts. This notion is consistent with the observation that Tcf2 expression does not depend on Sox9 activity (Dubois et al., 2011).

Additional insight into how Sox9 might control properties of ductal epithelial cells emerges from our intriguing discovery that Sox9 regulates expression of the Wnt co-receptor Lrp6 (Logan and Nusse, 2004) in pancreatic ducts. Because loss of *Lrp6* and defects in Wnt signal transduction are known to cause polycystic kidney

disease and ciliopathies (Lancaster et al., 2009; Logan and Nusse, 2004; Pinson et al., 2000), it is possible that disrupted Wnt signaling contributes to cyst formation in *Sox9*-deficient pancreas. Interestingly, *Lrp6* null mutant mice also display defects in Notch signaling during somitogenesis (Kokubu et al., 2004), suggesting a possible link between the Wnt and Notch pathways during ciliogenesis. Our findings now pave the way for future investigation of possible interactions between these signaling pathways in pancreatic ductal cell differentiation and maintenance.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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