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# Myt1 and Ngn3 form a feed-forward expression loop to promote endocrine islet cell differentiation

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ABSTRACT

High levels of *Ngn3* expression in pancreatic progenitor cells are both necessary and sufficient to initiate endocrine differentiation. While it is clear that the Notch-Hes1-mediated signals control the number of *Ngn3*-expressing cells in the developing pancreas, it is not known what factors control the level of *Ngn3* expression in individual pancreatic cells. Here we report that *Myt1b* and *Ngn3* form a feed-forward expression loop that regulates endocrine differentiation. Myt1b induces glucagon expression by potentiating *Ngn3* transcription in pancreatic progenitors. Vice versa, Ngn3 protein production induces the expression of *Myt1*. Furthermore, pancreatic *Myt1* expression largely, but not totally, relies on *Ngn3* activity. Surprisingly, a portion of *Myt1* expressing pancreatic cells express glucagon and other α cell markers in *Ngn3* nullizygous mutant animals. These results demonstrate that Myt1b and Ngn3 positively regulate each other's expression to promote endocrine differentiation. In addition, the data uncover an unexpected *Ngn3* expression-independent endocrine cell production pathway, which further bolsters the notion that the seemingly equivalent endocrine cells of each type, as judged by hormone and transcription factor expression, are heterogeneous in their origin.
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## Introduction

Endocrine islet cell differentiation requires interactions among multiple factors. Of particular importance for islet differentiation is the basic–helix–loop–helix protein Neurogenin 3 (Neurog 3 or Ngn3) (Sommer et al., 1996). *Ngn3* inactivation has been reported to totally abolish endocrine differentiation (Gradwohl et al., 2000). Ngn3 controls the expression of a cohort of genes that are important for endocrine differentiation (Gasa et al., 2004; Petri et al., 2006), including *Arx* (Collombat et al., 2003), *IA1* (Gierl et al., 2006; Mellitzer et al., 2006), *Hlxb9* (Li et al., 1999), *MafA* (Matsuoka et al., 2004), *MafB* (Artner et al., 2007), *NeuroD*//beta2 (*NeuroD1*) (Naya et al., 1997), *Nkx2.2* (Sussel et al., 1998), *Nkx6.1*, *Nkx6.2* (Henseleit et al., 2005; Sander et al., 2000), *Pax4* (Sosa-Pineda et al., 1997), *Pax6* (Sander et al., 1997; St-Onge et al., 1997), and many others (Petri et al., 2006; White et al., 2008). Ectopic *Ngn3* expression in early embryonic endodermal cells induces precocious endocrine differentiation (Apelqvist et al., 1999; Grapin-Botton et al., 2001; Schwitzgebel et al., 2000), possibly by inducing the expression of its downstream target, *NeuroD1*, which itself can induce endocrine differentiation when ectopically expressed (Ahnfelt-Ronne et al., 2007b; Gasa et al., 2004; Schwitzgebel et al., 2000). The well-orchestrated activation of these factors is critical for generation of the four major endocrine cell types, α, β, δ, and PP, that produce glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively.

In the mouse, endocrine cells are produced through two phases (Jensen et al., 2000a). From E9–E13.5 (referred to as wave I endocrine differentiation or primary transition), glucagon expressing α cells account for the majority of the differentiated endocrine cells. After E13.5 (referred to as wave II endocrine differentiation or secondary transition), all endocrine cell types are produced in significant numbers (Johansson et al., 2007; Jorgensen et al., 2007). The wave I endocrine cells do not contribute significantly to the mature endocrine cell mass (Herrera, 2000; Jensen et al., 2000a), yet their production shares similar molecular mechanisms with the wave II cell production. For example, loss of *Ngn3* function is reported to abolish both wave I and wave II endocrine differentiation (Gradwohl et al., 2000), although *Pdx1* is not required for wave I α cell production at all (Jonsson et al., 1995; Offield et al., 1996).

The key role of *Ngn3* necessitates its tight expression control for proper endocrine differentiation. $Ngn3^+$ (Ngn3-positive) cells are present in all embryonic stages between E9 and E18.5 in the developing mouse pancreas (Apelqvist et al., 1999; Jensen et al., 2000a; Jorgensen et al., 2007; Schwitzgebel et al., 2000). Yet each pancreatic cell only transiently expresses *Ngn3* in a shorter-than-48-hour time frame (Gu et al., 2002; Schwitzgebel et al., 2000). Thus, three distinct, yet related,

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aspects of *Ngn3* expression must be tightly regulated. First, the number of *Ngn3*-expressing cells must be controlled to ensure a balance between islet cell differentiation and progenitor cell proliferation (Apelqvist et al., 1999; Jensen et al., 2000b). Second, *Ngn3* expression must reach a threshold level to trigger endocrine differentiation (Wilson et al., 2003). Third, *Ngn3* expression need to be down-regulated as the endocrine differentiation program is switched on.

It is well established that Notch-mediated signaling regulates the number of $Ngn3^+$ cells. In pancreatic progenitors, active Notch signaling repress *Ngn3* expression by maintaining high levels of Hes1 (Apelqvist et al., 1999; Jensen et al., 2000b). Stochastic down-regulation of Notch signaling (Kaern et al., 2005) may then reduce *Hes1* expression level and subsequently leads to the activation of *Ngn3* in a subset of progenitor cells. Lateral inhibition of Notch signaling prevents the neighboring cells from activating *Ngn3* to restrict $Ngn3^+$ cell numbers (Bertrand et al., 2002). It is less clear how *Ngn3* is activated after cells are released from Hes1 repression (Lee et al., 2001). Complicating this issue is the finding that Ngn3 binds its own promoter to repress its transcription (Smith et al., 2004). While this self-inhibitory loop can help explaining the transient nature of *Ngn3* expression in specific islet progenitor cells, it will also prevent the high levels of Ngn3 activity required for endocrine commitment (Duvillie et al., 2006). It is therefore likely that other molecules act in concert with Ngn3 to inhibit the negative influence Ngn3 has on its own promoter. HNF6 directly binds the *Ngn3* promoter to activate *Ngn3* transcription and inactivation of HNF6 significantly reduces *Ngn3* expression (Jacquemin et al., 2000). However, HNF6 alone is not sufficient to induce high levels of *Ngn3* expression (Lee et al., 2001). HNF1 and HNF3 are shown to bind the *Ngn3* promoter, but it is not clear whether these two factors activate *Ngn3* expression *in vivo* (Lee et al., 2001). Thus, it is likely that other transcription factor(s) are required to augment the levels of Ngn3 protein in endocrine progenitors.

The zinc finger protein Myt1b (or Nzf2b) is a transcription factor that could potentially activate *Ngn3* expression. Myt1b is one of two proteins produced from the *Myt1* locus [Myt1a (Myt1) and Myt1b] (Gu et al., 2004; Matsushita et al., 2002). Myt1b has seven C2HC-type zinc fingers and a putative transcriptional regulatory domain. Myt1a is identical to Myt1b except that it is shorter in its N-terminus and has 6 zinc fingers (Matsushita et al., 2002; Romm et al., 2005; Wang et al., 2007). In all tissues examined, *Myt1b* is the predominantly expressed isoform (Gu et al., 2004; Matsushita et al., 2002). *Myt1* has two paralogs, *Myt1l* and *Myt3 (st18)* (Armstrong et al., 1997; Blasie and Berg, 2000; Kim et al., 1997; Kim and Hudson, 1992; Nielsen et al., 2004; Romm et al., 2005; Yee and Yu, 1998). These genes are highly expressed in developing neural tissues (Kim et al., 1997; Lein et al., 2007; Matsushita et al., 2002), and could behave either as transcriptional activators or repressors in a cell-context dependent manner (Bellefroid et al., 1996; Romm et al., 2005). In *Xenopus*, *xMyT1* and *xNgnR1* (an ortholog of Ngn3) synergistically promote neurogenesis (Bellefroid et al., 1996). In the embryonic pancreas, only *Myt1* is expressed at a detectable level in both endocrine progenitors and differentiated, hormone-producing cells (Gu et al., 2004). Loss of *Myt1* function partially impairs endocrine cell differentiation and result in glucose intolerance in pancreas-specific *Myt1* nullizygous animals (Wang et al., 2007). Furthermore *Myt1* expression partially depends on the activities of *Nkx6.1* and/or *Nkx6.2* gene products (Henseleit et al., 2005; Nelson et al., 2007). These data suggest that Myt1 has a role in endocrine cell differentiation. However, because *Myt1l* and *Myt3* expression is activated in the $Myt1^{-/-}$ pancreas, possibly compensating for loss of *Myt1* function, it remains obscure how Myt1 regulates endocrine differentiation (Wang et al., 2007). Particularly, it is not clear whether Myt1b functions upstream or downstream of *Ngn3* (Ahnfelt-Ronne et al., 2007a; Gu et al., 2004; Wang et al., 2007) and whether Myt1b acts as a transcription repressor or activator during isletogenesis (Bellefroid et al., 1996; Romm et al., 2005).

Here we describe the genetic interactions between *Myt1* and *Ngn3* using both loss-of-function and gain-of-function analyses in mouse tissues. We demonstrate that Myt1b induces precocious endocrine differentiation in a *Pdx1*-independent, but *Ngn3*-dependent, manner. Pancreatic *Myt1* expression largely, but not totally, relies on *Ngn3* activation. Significantly, we find that Myt1b up-regulates *Ngn3* expression in pancreatic progenitors and that Ngn3 can induce *Myt1* expression in both mouse cells *in vivo* and cell culture *in vitro*. Unexpectedly, glucagon-expressing cells are constantly detected in *Ngn3* deficient embryonic pancreata, and most of these cells co-express *Myt1*. Finally, we demonstrate that the transcriptional activation property of Myt1 is required for endocrine differentiation, suggesting that Myt1 functions as a transcriptional activator. These findings suggest that Myt1 and Ngn3 form a feed-forward expression loop to promote endocrine differentiation.

## Materials and methods

### *Mouse strains and care*

Mouse production and care follow standard protocols approved by the Vanderbilt Medical Center IACUC. For embryonic staging, the noon of vaginal plug appearance was counted as embryonic day 0.5 (E0.5). For routine mouse embryo production, the CD1 mouse strain was utilized (Charles River Laboratories, Inc., Wilmington, MA). For transgenic mouse production, B6/D2 mice were used (Charles River Laboratories, Inc. Wilmington, MA). Subsequent strain maintenance and crosses utilized CD1 mice. The $Pdx1^{tTA}$ mouse strain was described before (Holland et al., 2002). Genotyping follows published methods.

### *DNA constructs and transgenic animal derivation*

In order to ectopically express *Myt1b*, a minigene that contained intron # 6 of the Myt1b transcript was constructed using PCR and conventional molecular cloning. The final construct includes the full *Myt1b* open reading frame with minimal 5′UTR and 3′ UTR. In order to use the Teto promoter to drive gene expression, we PCR-amplified the TetO-CMV promoter from pTRE2 (Clontech, Paolo Alto, CA) and inserted in front of the *Myt1b* minigene. A SV40 PolyA signal was then inserted at the 3′ end of the *Myt1b* minigene to complete the construction. A similar approach was utilized for construction of tetO-Ngn3. Transgenic animal production followed standard pronuclear injection method. Genotyping utilized PCR-based technique. $Myt1b^{tet}$ mouse lines use oligos 5′-GCGTGTACGGTGGGAGGCCTATAT-3′ and 5′-ACTCTGTAAGCTTCGATGTCTGGA-3′. Expected fragment: 360 nt. $Ngn3^{tet}$ utilizes oligos 5′-GCGTGTACGGTGGGAGGCCTATAT-3′ and 5′-GGGTGGAATTGGAACTGAGCACT-3′. Expected fragment: 300 nt.

In order to produce the *Myt1b–Vp16* and *Myt1b–EnR* constructs, the cDNA coding for the second and third zinc fingers of Myt1b was PCR-amplified with two oligos: 5′-GATCCTTCCAGGGTGGAGAAG-3′ and 5′-GCTTCTCATGAGATTTGGCTAAT-3′. The DNA fragment was fused in frame with cDNAs encoding Vp16 (aa400–488) or EnR (2–298) protein (Muhr et al., 2001) to produce the desired constructs. In order to generate the *Myt1b* luciferase reporter construct, the putative mouse *Myt1b* promoter, including a conserved 1.8 kb element, was PCR-amplified and cloned upstream of a luciferase reporter. The construct was transfected together with a *Ngn3* expression vector into two well-established cell lines, the 3T3 fibroblast and P19 carcinoma cell lines. The oligos used were: forward: 5′-TTGTAGCACATATTGGCTTCTCC-3′. Reverse: 5′-GCTCCAATATATCTGTTTACTC-3′. The PCR fragment was cloned into the PGL3 luciferase reporter vector (Promega, Madison, WI). The NeuroD-luciferase construct was described previously (Huang et al., 2000). Luciferase assay followed standard protocol (Williams et al., 1989).

### *Ngn3 targeting*

The *Ngn3* locus targeting followed a standard protocol. The targeting vector contains the following features sequentially: 5.3 kb *Ngn3* 5′arm-LoxP-*Ngn3* open reading frame-FRT-pGK-neo-FRT-LoxP-$CreER^T$-1.3 kb *Ngn3* 3′arm. The 5′arm and 3′arm were obtained from 129svev genomic DNA using long range PCR. The Primers utilized: 5′arm forward: 5/-GGGTGTTATATGTGTGCCAAGGGCT-3′, 5′arm reverse: 5/-CTGCGGTTGGGAAAAAATG-GAAGGTGT-3′. 3′ arm forward: 5′-GAAGCTCCGCGCCCGACGCGGAGGGC-3′, 3′ arm reverse: 5′-CTTGTGAAGATTCTCGACGTTCATC-3′. The DNA vector, containing a FRT-flanked pGK-neo selection cassette and DTA for selection, is previously described (Wang et al., 2007). DNA electroporation utilized TL1 ES cells (Wang et al., 2007). ES cell selection and blastocyst injection follow standard methods. Both Southern blot and PCR-based assays were used to select targeted ES cells clones. Southern blot utilized a DNA fragment amplified by oligos 5′-CTAGAATGCCTAGGAAGAGGAA-3′ and 5′-CCACAT-CATGGCAGTGTGTCCTGA-3′ as probe. Oligos used for PCR-based genotyping: 722: 5′-CTATCCACTGCTGCTTGTCACTG-3′. 723: 5′-TGTGTCTCTGGGGACACTTGGAT-3′. jv45: 5′-TTCCGGTTATTCAACTTGCACC-3′. The $Ngn3^-$ allele produces a 350 bp band with (722 + Jv45) but eliminates the wild type 219 bp band amplified with (722 +723).

*Chicken in ovo endoderm electroporation*

Chicken electroporation was previously described (Ahnfelt-Ronne et al., 2007a; Grapin-Botton et al., 2001; Gu et al., 2004). Briefly, fertilized White Leghorn eggs (Triova, Denmark) were incubated at 38 °C for 55 h. Electroporation was performed on chicken embryos at HH st. 10–15 (Hamburger and Hamilton, 1992). The DNA with a fixed concentration of 2 μg/μl in PBS (with 1 mM $MgCl_2$, 3 mg/ml carboxymethylcellulose, 0.66 mg/ml Fast Green) was injected into the blastocoels of windowed eggs. Three to five square 50 ms pulses of 7–15 V were applied from a BTX ECM830 electroporator. The eggs were sealed and allowed to develop for 52–72 h at 38 °C. After incubation, the whole embryo was fixed. The gut derived tissues were then collected for gene expression analyses (Ahnfelt-Ronne et al., 2007b).

*Immunohistochemistry/immunofluorescence*

Immunofluorescence/immunohistochemistry followed established protocols. Tissues were stained either as frozen sections or paraffin sections. Whole mount immunofluorescence strictly followed the protocol depicted in Ahnfelt-Ronne et al. (2007b). For frozen sections, dissected tissues were immediately frozen in OCT and sectioned when needed. Sectioned tissues were collected onto Superfrost-plus slides, left at room temperature for 30 min, and fixed in 4% paraformaldehyde at room temperature for 15 min. Slides were washed in PBS, permealized in 0.1% triton X-100, and immunostained. For paraffin section, mouse tissues were fixed in 4% paraformaldehyde overnight at 4 °C or 4 h at room temperature. They would then be prepared for section following routine procedures. Primary antibodies used were: guinea pig anti-glucagon (Dako, Carpinteria, CA), Rabbit anti-MafB, gifts from R. Stein (Matsuoka et al., 2004), goat anti-Pdx1, a gift from C. Wright, guinea pig anti-Ngn3, a gift from M. Sander (Seymour et al., 2007), rabbit anti-Ngn3 (Gu et al., 2002), rabbit anti-Myt1 (Wang et al., 2007), rabbit anti-Foxa2 (Santa Cruz Biotechnologies, Santa Cruz, CA), mouse anti-Nkx6.1 and mouse anti-Pax6 (Development Hybridoma Bank, University of Iowa, Iowa, IA), rabbit anti-PC1/3 (Chemicon, Temetula, CA). Secondary antibodies used were: FITC-conjugated donkey anti-rabbit IgG; Cy3-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-guinea pig IgG, Cy3-conjugated donkey anti-goat, and Cy5-conjugated donkey anti-rabbit (Jackson Immunoresearch, West Grove, PA). All antibodies utilized a1:500–1:2000.

*Microscopy and statistical analysis*

All fluorescent images were obtained using confocal microscopy. For quantification of glucagon expression and *Myt1b* ectopic expression, samples were frozen and sectioned at 15 μm intervals. All sections were then collected from individual sample and stained for hormone and transcription factor expression. Confocal optical sections were taken at 0.4–0.6 μm intervals on all stained tissues. For Myt1b level estimation in each cell, all optical sections for individual nucleus are projected to one picture. The fluorescence intensity within each nucleus was then compared using BIOQUANT true-color windows system (R&M Biometrics, Nashville, TN). For quantification of *Nkx6.1* and *MafB* expression, paraffin-sections at 6 μm intervals were utilized. In this case, only one optical section of each tissue section was counted. Statistical analyses utilized standard Student *T* test. A *P*-value of 0.05 or better was considered significant. All quantification data are presented as the mean±standard error over the mean.

## Results

*Derivation of transgenic mouse lines that express Myt1b and Ngn3 in a tTA-dependent fashion*

Our previous studies of ectopic gene expression in chicken embryonic endodermal cells suggest that Myt1b induces endocrine differentiation (Ahnfelt-Ronne et al., 2007a; Gu et al., 2004). Yet our inability to precisely control the timing and protein level of ectopic gene expression in chicken embryos led to a considerable variability in endocrine induction efficiency. In addition, loss of gene function is not achievable in chicken tissues. We therefore combined studies in mouse and chicken to determine how Myt1b interacts with Ngn3 to induce endocrine differentiation.

We first examined the Myt1b–Ngn3 interaction by a gain of function approach. We used the well-established, inducible TetOff/TetOn system to regulate *Myt1b* and *Ngn3* ectopic expression in pancreatic cells (Gossen et al., 1994). Specifically, we derived transgenic mouse lines that utilized a TetO-CMV synthetic promoter to control the expression of *Myt1b* or *Ngn3* (*Myt1b*$^{tet}$ and *Ngn3*$^{tet}$). The expression of *Myt1b* and *Ngn3* in these animals could be activated by a transactivator, tTA, in the absence of tetracycline or doxycycline (Dox) (Gossen et al., 1994). This approach avoided the possible lethality caused by uncontrolled ectopic *Myt1b* or *Ngn3* expression in pancreatic cells and allowed for the derivation of stable mouse lines, in which Myt1b or Ngn3 production could be activated by providing active tTA in desired cell types at specific stages. We chose a knockin mouse line that expresses tTA under the control of the *Pdx1* promoter (Holland et al., 2002) to activate *Myt1b* or *Ngn3* ectopic expression in pancreatic cells. We expected the compound *Myt1b*$^{tet}$; *Pdx1*$^{tTA/+}$ and *Ngn3*$^{tet}$; *Pdx1*$^{tTA/+}$ animals would ectopically express *Myt1b* and *Ngn3* in most, if not all, of the Pdx1$^+$ progenitors without Dox.

Seven *Myt1b*$^{tet}$ and nine *Ngn3*$^{tet}$ independent transgenic mouse lines were derived through pronuclear injection. As expected, none of these animal lines ectopically expressed the transgene in the absence of tTA (data not shown). One of the *Myt1b*$^{tet}$ transgenic lines (*Myt1b*$^{tet2}$), when

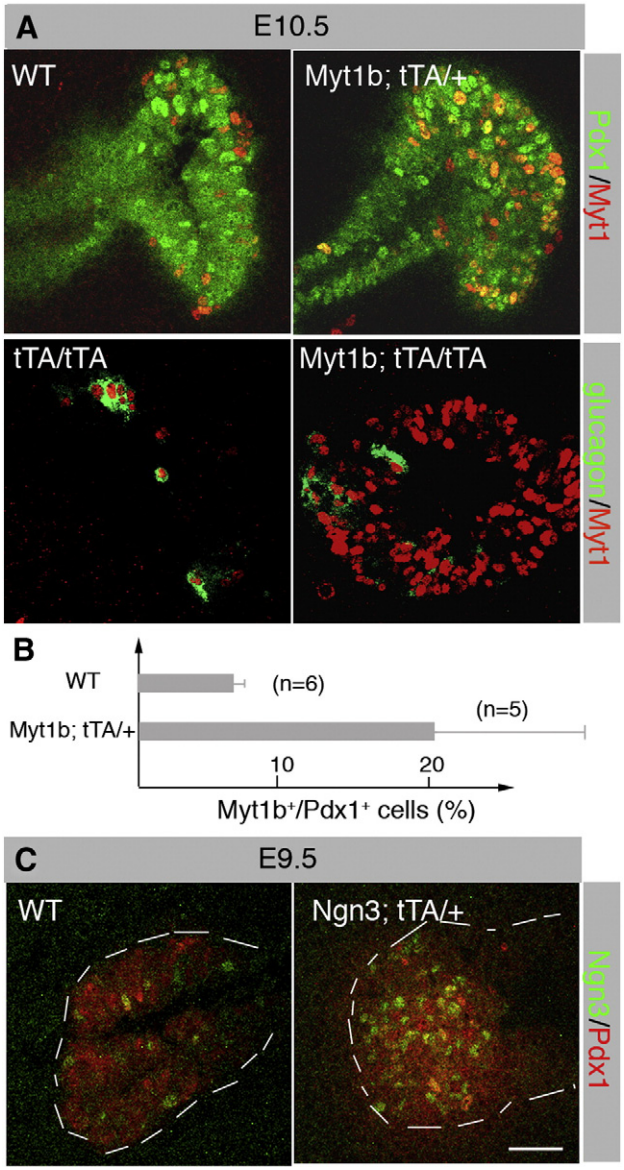


**Fig. 1.** Derivation of mouse lines that ectopically express *Myt1b* and *Ngn3*. (A) Pancreatic *Myt1b* expression (red) at E10.5. Wild type (WT), which showed identical *Myt1* expression pattern as *Pdx1*$^{tTA/+}$ pancreas, and *Pdx1*$^{tTA/tTA}$ (labeled as tTA/tTA) pancreata were used as controls. The presence of *Myt1b*$^{tet2}$ (labeled as *Myt1b*) led to *Myt1b* ectopic expression in the presence of tTA. Pdx1 (green) or glucagon staining (green) were used to mark the position of the pancreatic buds. Note that Myt1 antibodies only stain frozen, lightly fixed sections, on which the Pdx1 signal appears diffuse. (B) Quantification of Myt1b ectopic expression in control and *Myt1b; Pdx1*$^{tTA/+}$ pancreata. The number of Myt1b$^+$ cells is normalized against the number of Pdx1$^+$ cells. (C) *Ngn3* expression (green) in WT and *Ngn3*$^{tet8}$; *Pdx1*$^{tTA}$ (labeled as Ngn3; tTA) pancreata. White broken lines, pancreatic region. Note the apparent thickening of pancreatic epithelium that ectopically expresses *Ngn3*. All panels used identical scales, bar=20 μm.

combined with *Pdx1*$^{tTA}$ in the absence of Dox, produced Myt1b protein in 20.5±9.5% ($n$=6) of the Pdx1$^+$ cells in the pancreas (Figs. 1A and B). By comparison, only 7.3±0.8% ($n$=5) of Pdx1$^+$ cells produced detectable Myt1 in the *Pdx1*$^{tTA/+}$ control pancreas. Significantly, pancreatic Myt1b production in *Myt1b*$^{tet}$; *Pdx1*$^{tTA/+}$ individuals and wild type littermates appeared at comparable levels on per cell basis (Fig. 1A), as judged by fluorescence intensity within each nuclei during side-by-side immunofluorescence staining (see Materials and Methods). We also examined Myt1 production in *Myt1b*$^{tet}$; *Pdx1*$^{tTA/tTA}$ pancreata, in which no functional Pdx1 existed, yet more tTA was expected to be produced within each cell (Holland et al., 2002). *Myt1* was ectopically expressed in most, if not all, of the cells in the *Myt1b*$^{tet}$; *Pdx1*$^{tTA/tTA}$ prospective pancreatic epithelium, suggesting that a higher Myt1b production could be achieved by increasing the *Pdx1*$^{tTA}$ dosage (Fig. 1A). We explored whether Myt1b production could be activated at other developmental stages by controlling the availability of Dox during embryonic development. Plugged female animals were fed with Dox only between E7.5 and E12.5. Myt1b production was then characterized at E15.5 and E17.5 in *Myt1b*$^{tet}$; *Pdx1*$^{tTA/+}$ pancreata. We found that this manipulation could not drive ectopic *Myt1b* expression in more pancreatic cells than in control littermates, other than a few individual differentiated islet cells seemed to have a higher level of Myt1b protein (Supplementary Fig. 1). Similarly, when we fed plugged females with Dox between E7.5 and E18.5, we could not detect significant *Myt1b* over-expression in the Pdx1-expressing postnatal cells either (data not shown). This deficiency prevented us from studying the effect of *Myt1b* ectopic expression in a temporally controlled manner. The remaining six *Myt1*$^{tet}$ mouse lines did not show detectable ectopic gene expression when combined with *Pdx1*$^{tTA}$ and were not maintained.

Seven *Ngn3*$^{tet}$ transgenic lines showed wide spread ectopic *Ngn3* expression when combined with the *Pdx1*$^{tTA}$ allele in early embryonic pancreas (Fig. 1C and data not shown). Consistent with published data that ectopic *Ngn3* expression induces precocious endocrine differentiation, nearly all pancreatic cells in the *Ngn3*$^{tet}$; *Pdx1*$^{tTA/+}$ animals were converted to glucagon-expressing cells after E11.5 (data not shown). Most of the Pdx1$^+$ cells in the *Ngn3*$^{tet}$; *Pdx1*$^{tTA/+}$ animals appeared to have delaminated from the developing epithelium, consistent with previous findings that Ngn3 induces epithelial-to-mesenchymal transition (Apelqvist et al., 1999; Grapin-Botton et al., 2001; Schwitzgebel et al., 2000). One of these transgenic lines, *Ngn3*$^{tet8}$, that produced Ngn3 protein in a comparable level to that of wild type littermates, on a per cell basis, was used for the following studies (Fig. 1C).

### *Myt1b is sufficient to induce endocrine differentiation in pancreatic cells*

*Myt1b*$^{tet2}$; *Pdx1*$^{tTA/+}$ animals were derived by standard genetic crosses in the absence of Dox. This allowed for *Myt1b* ectopic expression in pancreatic progenitors as soon as *Pdx1* expression was turned on (Holland et al., 2002). Glucagon expression was examined to determine whether Myt1b was sufficient to induce endocrine differentiation at several stages (E10.5, E11.5, and E12.5). Ectopic *Myt1* expression significantly increased the number of glucagon$^+$ cells in the developing pancreas at E11.5 (Figs. 2A–C and data not shown). The expression of insulin, pancreatic polypeptide, and somatostatin was not appreciably altered (data not shown). In order to examine whether sustained Myt1b production at stages later than E12.5 enhances endocrine differentiation, we examined hormone expression in *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/+}$ and *Pdx1*$^{tTA/+}$ control pancreata, in the absence of Dox, at E17.5 and E18.5. At both stages, *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/+}$ pancreata did not produce significantly more hormone-expressing cells than control littermate. This result is consistent with the finding that ectopic *Myt1b* expression could not be activated at stages after E13.5 (Supplementary Fig. 1), making it impossible to determine whether Myt1b is sufficient to induce endocrine differentiation at later embryonic stages.

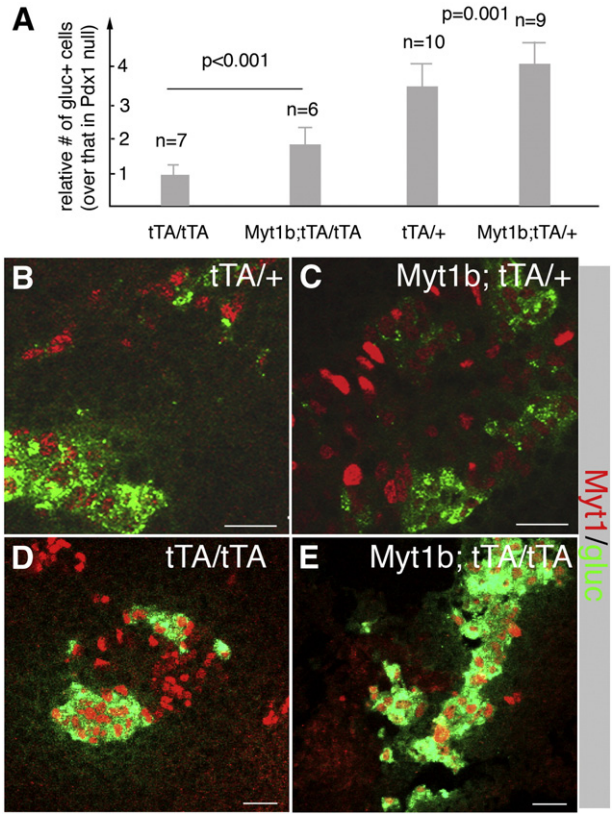


**Fig. 2.** Ectopic Myt1b expression induces glucagon expression. (A) The relative number (mean±S.E.M.) of glucagon-expressing cells in E11.5 embryos (tTA=Pdx1$^{tTA}$. Myt1b=Myt1b$^{tet2}$). (B–E) Myt1 and glucagon (gluc) production in representative sections of E11.5 pancreata. Scale bars=20 μm.

We examined glucagon production in the *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ animals to determine whether Myt1b induces endocrine differentiation in a Pdx1-dependent pathway. E11.5 *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreata had 1.8 fold more glucagon$^+$ cells than *Pdx1*$^{tTA/tTA}$ littermates (Figs. 2A, D, and E), suggesting that Myt1b is sufficient to induce endocrine differentiation in the complete absence of Pdx1. Unlike endodermal cells that ectopically expressed *Ngn3*, which largely differentiated to endocrine cells (Apelqvist et al., 1999; Schwitzgebel et al., 2000), only about half of the Myt1$^+$ cells ($n$=6) in the *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreas turned on glucagon expression (Figs. 2B–E). By E18.5, only rare glucagon$^+$ cells could be detected in *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreata. This finding suggests that without Pdx1-dependent cell expansion or survival (Jonsson et al., 1994; Offield et al., 1996), early glucagon$^+$ cells might have died or have trans-differentiated into other cell types, possibly due to the lack of paracrine signals produced by Pdx1$^+$ cells or other unknown reasons.

Because only one *Myt1b*$^{tet}$ transgenic mouse line could produce ectopic Myt1b protein in pancreatic progenitors, we verified our above findings by generating other transgenic mouse lines that ectopically expressed *Myt1b*. We derived *Pdx1–Myt1b*$^{tg}$ mice, in which *Myt1b* was ectopically expressed under the control of a 4.3 kb *Pdx1* promoter. This promoter fragment is known to drive transgene expression in a pattern similar to that of endogenous *Pdx1* (Wu et al., 1997). Three *Pdx1–Myt1b*$^{tg}$ embryos that expressed ectopic *Myt1b* were obtained. In these pancreata, a 2.3 fold increase in glucagon$^+$ cells was observed. Similar to *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreata, many Myt1b$^+$ cells in *Pdx1–Myt1b*$^{tg}$ pancreata failed to activate glucagon expression (Supplementary Fig. 2). These findings demonstrate that Myt1b promotes endocrine differentiation, yet Myt1b has a weaker endocrine inducing capability than Ngn3.

Because Myt1b was also produced in some stomach and duodenal cells in *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/+}$ and *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ animals, we examined whether endocrine differentiation could be induced by *Myt1b* ectopic expression in these non-pancreatic cells at E10.5 and E12.5. The number of hormone (glucagon, insulin, SS and PP) expressing cells remained identical in the duodenal and stomach tissues in animals that did or did not ectopically express *Myt1b* (data not shown). These data demonstrate that ectopic Myt1b production cannot convert specified duodenal and stomach cells into endocrine islet cells.

### *Ectopic Myt1b-induced glucagon-expressing cells are immature α cells*

Endocrine islet cell production in the mouse embryonic pancreas occurs in two phases. From E9–E12.5, mostly glucagon expressing α cells are produced (Jensen et al., 2000a). The production of these cells does not require *Pdx1* activity and these cells might not contribute significantly to adult islet pool (Herrera, 2000; Offield et al., 1996). These early glucagon-expressing cells are therefore considered immature endocrine cells and are shown to express the protein convertase, PC1/3 (Wilson et al., 2002). After E12.5, all four endocrine cell types are produced and the mature α cells switch off PC1/3 expression.

We determined whether the Myt1b-induced endocrine cells were mature α cells by analyzing their expression of PC1/3 and other known α cell markers, including MafB (Artner et al., 2007) and Nkx6.1 (Henseleit et al., 2005). Most, if not all, glucagon expressing cells in the *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/+}$ or the *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreata (E12.5) produced high levels of PC1/3 (Supplementary Fig. 3A and Fig. 3A), suggesting that induced glucagon-expressing cells, regardless of the presence or absence of Pdx1, are immature α cells. In addition, *MafB* expression was observed in nearly all glucagon$^+$ cells in the *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/+}$ or *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreas as in control littermates (Supplementary Fig. 3B and Fig. 3B), suggesting that Myt1b is sufficient to activate *MafB* expression. Interestingly, ectopic *Myt1* expression significantly increased the number of Nkx6.1$^+$ pancreatic cells: at E12.5, there was a 273 ± 160% increase of Nkx6.1$^+$ cells in the *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreata over that of the *Pdx1*$^{tTA/tTA}$ littermates (Figs. 3C and D). Because Nkx6.1/Nkx6.2 were reported to be required for strong *Myt1b* expression and α cell differentiation, this latter finding demonstrates a possible feedback activation link between *Myt1* and *Nkx6.1/Nkx6.2* transcription factors (Henseleit et al., 2005). This result is also consistent with the finding that *Nkx6.1* expression is maintained in the glucagon expressing cells of *Pdx1*$^{-/-}$ mutant pancreas (Pedersen et al., 2005).

### *Myt1b induces endocrine differentiation through Ngn3*

We next examined how Myt1 induces endocrine differentiation. Our published results suggest that Myt1b partially represses Notch signaling in endodermal cells, an essential process for *Ngn3* expression activation (Ahnfelt-Ronne et al., 2007a; Xu et al., 2006). We therefore examined whether Myt1b ectopic expression activates *Ngn3* expression. Embryos that ectopically expressed *Myt1b* were obtained at different stages, and pancreatic *Ngn3*-expressing cells were counted. At E10.25, the number of Ngn3$^+$ cells in *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/+}$ pancreata increased by 136 ± 22% over that of *Pdx1*$^{tTA/+}$ littermates (Fig. 4A and Supplementary Fig. 4), consistent with the findings that more glucagon-expressing cells were produced in pancreata with ectopic *Myt1b* expression. Enhanced *Ngn3* expression was observed in *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreas as well. At E9.5 and E10.25, *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreata contained visibly more Ngn3$^+$ cells than wild type littermates (Figs. 4B and 4C). Notably, glucagon$^+$Ngn3$^+$ cells were detected in *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreata, but not in control littermates (Fig. 4C). At E10.25, *Pdx1*$^{tTA/tTA}$ pancreas produced no more than two Ngn3$^+$ cells ($n$ = 10), yet *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ embryos produced an average of 12 Ngn3$^+$ cells per pancreas ($n$ = 10). These data suggest that Myt1b can induce *Ngn3* expression and promote endocrine differentiation, even in the absence of Pdx1.

Because Myt1 is expressed in both endocrine progenitor cells and differentiated adult islet cells (Wang et al., 2007), it is possible that Myt1 acts as a downstream target of *Ngn3* to mediate endocrine differentiation. In this case, Myt1 should induce endocrine differentiation in the absence of Ngn3. To investigate this possibility, we derived a targeted conditional *Ngn3* allele, which has the *Ngn3* coding region flanked by two LoxP sites to potentially produce a *Ngn3* null allele (Figs. 4D–F). Myt1b$^{tet2}$; *Pdx1*$^{tTA/tTA}$; *Ngn3*$^{-/-}$ embryos were obtained, such that *Myt1b* was ectopically expressed in *Ngn3* nullizygous background. Eliminating *Ngn3* activity nearly abolished glucagon expression with *Myt1b* ectopic expression (Fig. 4G). These data provide strong genetic evidence that *Ngn3* is required for Myt1b-induced endocrine differentiation, i.e. Myt1b induces endocrine differentiation through activation of *Ngn3*.

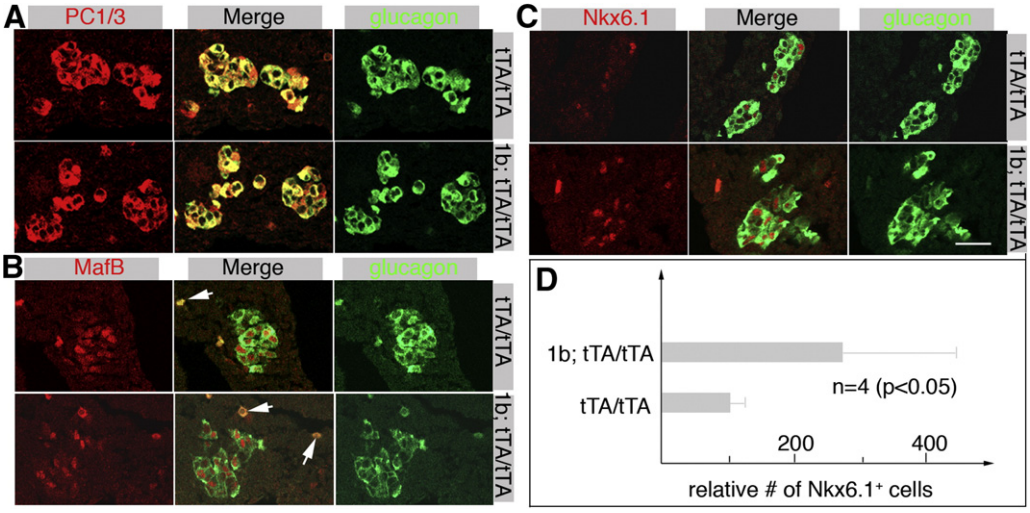


**Fig. 3.** *Myt1b* ectopic expression induces the expression of α cell markers. Sections of E12.5 pancreata. *Pdx1*$^{tTA/tTA}$ pancreata were used as controls. (A) Co-expression of glucagon and PC1/3 in Myt1b induced endocrine cells. (B and C) *MafB* and *Nkx6.1* expression in pancreata that ectopically express *Myt1b*, respectively. Each sample contains two single fluorescence channels and one merge. tTA/tTA = Pdx1$^{tTA/tTA}$. 1b;tTA/tTA = Myt1b$^{tet2}$; Pdx1$^{tTA/tTA}$. White arrows in panel B point to blood cells. All panels used identical scales, bar = 20 μm.

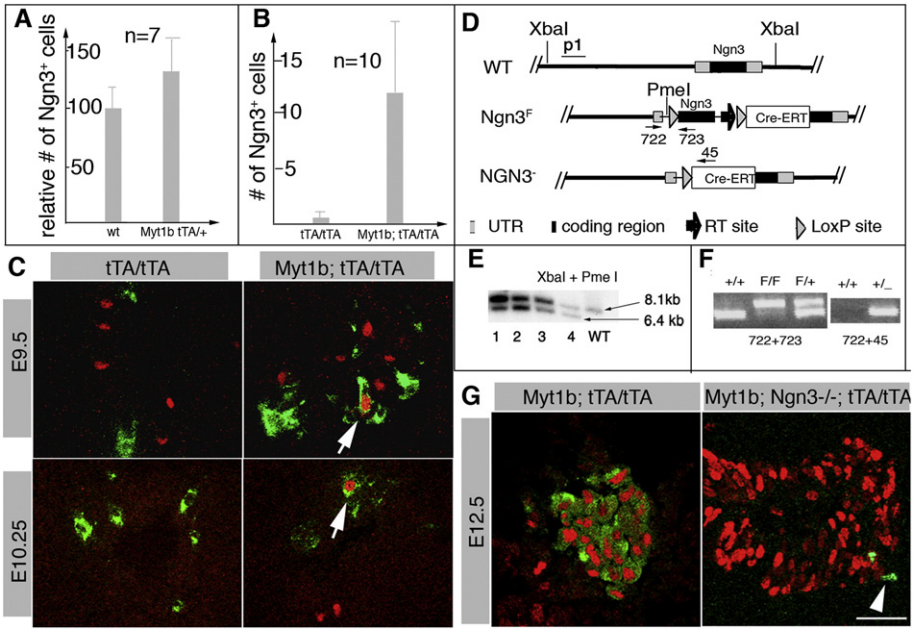


**Fig. 4.** Myt1b induces glucagon expression through *Ngn3*. Hemizygous pancreata containing *Pdx1*$^{tTA}$ and other mutations were utilized (tTA=Pdx1$^{tTA}$. Myt1b=Myt1b$^{tet2}$). (A) The relative number of Ngn3$^+$ cells in Myt1b; Pdx1$^{tTA/+}$ pancreata over that of wild type control littermates at E10.25 ($n$=7, $P$=0.015). (B) The average number of Ngn3$^+$ cells in *Pdx1*$^{-/-}$ pancreata that ectopically express Myt1b at E10.25. (C) Immunofluorescence showing Ngn3 production in pancreata that ectopically express *Myt1b*. Two stages, E9.5 and E10.25, are shown. Note the presence of glucagon$^+$Ngn3$^+$ cells (white arrows) in pancreata with ectopic *Myt1* expression. Red, Ngn3. Green, glucagon. (D–F) The production and genotyping strategies of a novel *Ngn3* null allele (*Ngn3*$^-$). (D) The structure of the *Ngn3* wild type, floxed (*Ngn3*$^F$) and null allele. A *Pme*I site was introduced in the 5′end of the *Ngn3* coding cDNA in the *Ngn3*$^F$ allele. Also note that the *Ngn3*$^-$ allele produces CreER™ a feature that is not used in this study. (E) Southern blot identifies four targeted ES cell clones, producing a 8.1 kb wild type and a 6.4 kb mutant band when digested with *Xba*I+*Pme*I and blotted with P1 probe. The numbers (45, 722, and 723) and small arrows indicate the position of oligos used for PCR-based genotyping (F). (G) Myt1b-induced glucagon expression in pancreata with or without *Ngn3* at E12.5. Note the rare glucagon$^+$ cells (green, white arrowhead) in *Ngn3*$^{-/-}$ mutant pancreas. Red, Myt1. All panels utilized identical scales, bar=20 μm.

*Myt1 expression largely, but not totally, depends on Ngn3*

We next examined whether endogenous *Myt1* expression totally depends on *Ngn3*. E10.5, E12.5, E13.5, and E15.5 *Ngn3* nullizygous pancreata were obtained. Pancreatic Myt1+ cells were detected in all stages examined (Fig. 5). The pancreatic identity of these Myt1$^+$ cells was confirmed by the surprising findings that a portion of these Myt1$^+$ cells co-expressed glucagon (Figs. 5A–C) and MafB (Fig. 5D). After E15.5, no glucagon$^+$ cells could be found in Ngn3$^{-/-}$ pancreas, although Pdx1$^+$Myt1$^+$ cells could still be detected (Supplementary Fig. 5). At all stages examined, the number of Myt1$^+$ cells represented less than 5% of that of the wild type littermate, demonstrating that *Myt1* expression is largely, but not totally, dependent on *Ngn3*. At present, we do not know whether these Myt1$^+$ cells depend on the activation of *Ngn3* paralogs, *Ngn1* and *Ngn2* (Sommer et al., 1996), in the *Ngn3* null background.

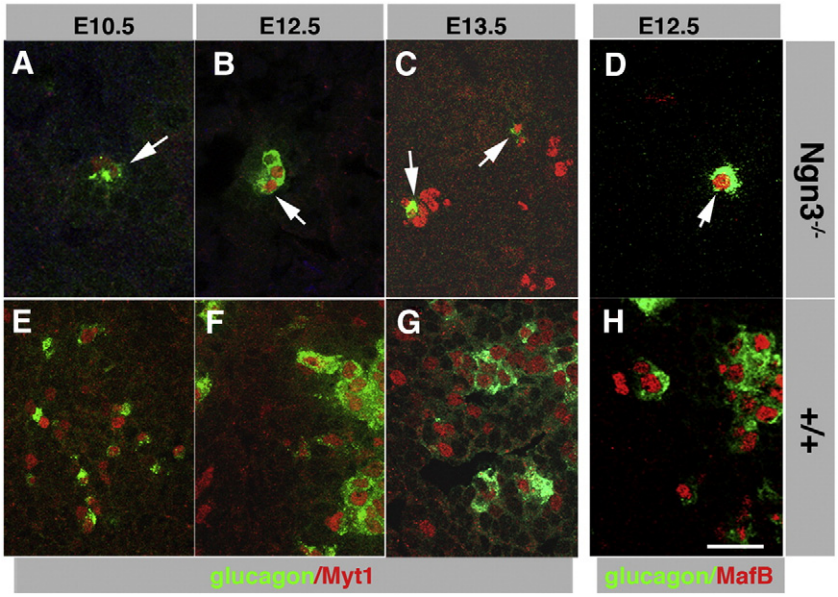


**Fig. 5.** *Ngn3*$^{-/-}$ mutant pancreas maintains *Myt1* and glucagon expression. Glucagon (green), *Myt1* or *MafB* (red) expression at three stages, E9.5, E12.5, and E13.5. (A–D) *Ngn3*$^{-/-}$ pancreata. (E–H) Wild type pancreata. White arrowheads point to glucagon$^+$Myt1$^+$ cells in *Ngn3*$^{-/-}$ pancreata. All panels used identical scales, bars=10 μm.

*Ngn3 induces Myt1 expression*

The dramatic reduction of *Myt1* expression in *Ngn3*$^{-/-}$ pancreata suggests that Ngn3 activates *Myt1* expression. Indeed, in the *Ngn3*$^{tet8}$; *Pdx1*$^{tTA/+}$ pancreata, where *Ngn3* was ectopically expressed in most, if not all, of the Pdx1$^{+}$ progenitors at early embryonic stages, Myt1 production was dramatically increased (Figs. 6A and B).

We further analyzed whether Ngn3 can activate the *Myt1b* promoter in tissue culture. Alignment of mouse, rat, human and chicken genomic DNA sequences upstream of the respective *Myt1b* transcription initiation site revealed several DNA blocks that are highly conserved in all four species (Fig. 6C), suggesting that these DNA elements constitute the promoter that regulates the pancreatic and/or neuronal *Myt1* expression. The putative mouse *Myt1b* promoter was utilized to drive a luciferase reporter gene in 3T3 fibroblast and P19 carcinoma cell lines. Ngn3 activated the *Myt1b* promoter in both cell lines (Figs. 6D and E). A mutant Ngn3 protein, which contained a N89D mutation that inactivated its DNA binding activity, could not activate the *Myt1b*-luciferase reporter (Fig. 6D).

*Myt1 is likely a transcriptional activator during endocrine differentiation*

A dominant negative Myt1b molecule represses endocrine differentiation in both mouse and chicken tissues (Ahnfelt-Ronne et al., 2007b; Gu et al., 2004). Loss of the endogenous *Myt1* function partially impairs endocrine differentiation and adult islet function (Wang et al., 2007). However, it is not clear whether *Myt1b* behaves as a transcriptional repressor or activator in the endocrine differentiation process. To test these possibilities, we created artificial reading frames that encoded a Myt1-activator and a Myt1-repressor protein, respectively. The Myt1-activator was a fusion between the second and third zinc fingers of Myt1b and the VP16 trans-activation domain and the Myt1-repressor was a fusion between the same zinc fingers and the EnR repressor domain (Fig. 7A). We expected that these proteins would possess activator or repressor activities that were inherent to VP16 and EnR, respectively (Bellefroid et al., 1996), yet would bind the same DNA elements recognized by Myt1b. If Myt1b behaves as a transcriptional activator during endocrine differentiation, we expect that the Myt1–VP16 protein to substitute Myt1 for inducing endocrine differentiation, whereas Myt1–EnR would repress endocrine differentiation. Vice Versa, if Myt1 behaves as a transcriptional repressor during endocrine differentiation, we expect that the Myt1–EnR protein to substitute Myt1 for inducing endocrine differentiation, whereas Myt1–VP16 to inhibit islet cell production.

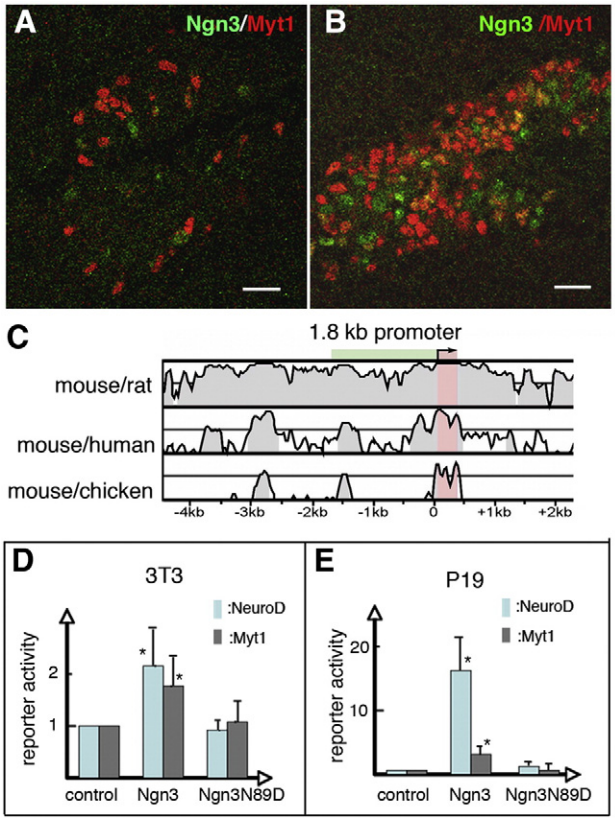


**Fig. 6.** Ectopic *Ngn3* expression induces *Myt1* expression. (A and B) *Myt1* and *Ngn3* expression in pancreata that ectopically express *Ngn3*. (C) Genomic pair-wise alignment of *Myt1* promoters from different species. Grey peaks indicate conserved regions. The pink column indicates the first exon. The 1.8 kb *Myt1b* promoter region used for reporter assays is marked by a light green bar on top. (D and E) *Myt1* promoter activity assays in 3T3 and P19 cell lines. In each cell line, mock transfected, *Ngn3*, and a mutant *Ngn3* (N89D) expressing vectors were co-transfected with the respective luciferase reporter plasmid. * denotes that the differences between control and experimental groups were statistically significant. Scale bars = 10 μm.

Chicken embryos were electroporated with *Ngn3* expressing vector alone or in combination with plasmids that expressed either of the two above fusion proteins. As previously reported (Grapin-Botton et al., 2001), *Ngn3* ectopic expression in chicken embryonic endodermal cells caused epithelial cells to delaminate from the gut tube and turned on the expression of endocrine markers such as glucagon and *Pax6* (Grapin-Botton et al., 2001). When *Ngn3* was expressed in combination with Myt1–VP16, both glucagon and Pax6 could be detected in the electroporated cells, suggesting that activation of *Myt1* target genes (by Myt1–VP16) has minimal effect on Ngn3-induced endocrine differentiation (Figs. 7B–D). In contrast, the presence of Myt1–EnR substantially reduced the differentiation induced by *Ngn3* ectopic expression (Figs. 7E–G). Although *Ngn3* and *Myt–EnR* vectors electroporated cells still delaminated from the endodermal epithelium, the number of glucagon positive cells was reduced and the electroporated cells appeared more scattered (compare Figs. 7F and 7C). Significantly, Myt1–EnR almost completely blocked the expression of *Pax6* induced by Ngn3 (compare Figs. 7G and D). These data demonstrate that converting Myt1b into a transcription repressor inhibits Ngn3-induced endocrine differentiation, i.e. Myt1b acts as a transcriptional activator for endocrine differentiation. Interestingly, Myt1–EnR did not prevent the epithelial cell from delaminating, suggesting that endocrine hormone expression and epithelial delamination are mediated by distinct pathways initiated by Ngn3 (Figs. 7B, C, E, and F).

## Discussion

Loss or attenuation of endocrine function, particularly that of the insulin-producing β cells, results in *diabetes mellitus*. Because replenishing lost β cells provides a potential cure for diabetes, understanding how endocrine islet cells develop *in vivo* and whether such mechanisms could be explored for ES cell-based cell production *in vitro* or for inducing β cell regeneration *in vivo* has attracted much attention in the past decade (Blyszczuk and Wobus, 2006; Bonner-Weir and Weir, 2005; Colman, 2004; Heit and Kim, 2004; Kaczorowski et al., 2002; Kania et al., 2004; Noguchi, 2007; Santana et al., 2006; Schroeder et al., 2006). To this end, identifying molecules capable of inducing endocrine cell differentiation in animal models and elucidating their mechanism of action are particularly valuable for diabetes research.

Here, we show that *Myt1* and *Ngn3* form a feed-forward expression loop to promote the differentiation of most, if not all endocrine cells. This finding is consistent with a simplified model where Myt1 activation provides an exit mechanism to break the *Ngn3* self-inhibitory loop. This allows the development of high levels of *Ngn3* expression required for endocrine commitment (Fig. 8). In particular, it is likely that during endocrine differentiation, a slight fluctuation in Notch activity, caused by stochastic gene expression (Kaern et al., 2005) and/or modification by the Fringe molecules (Xu et al., 2006), initiates *Myt1*

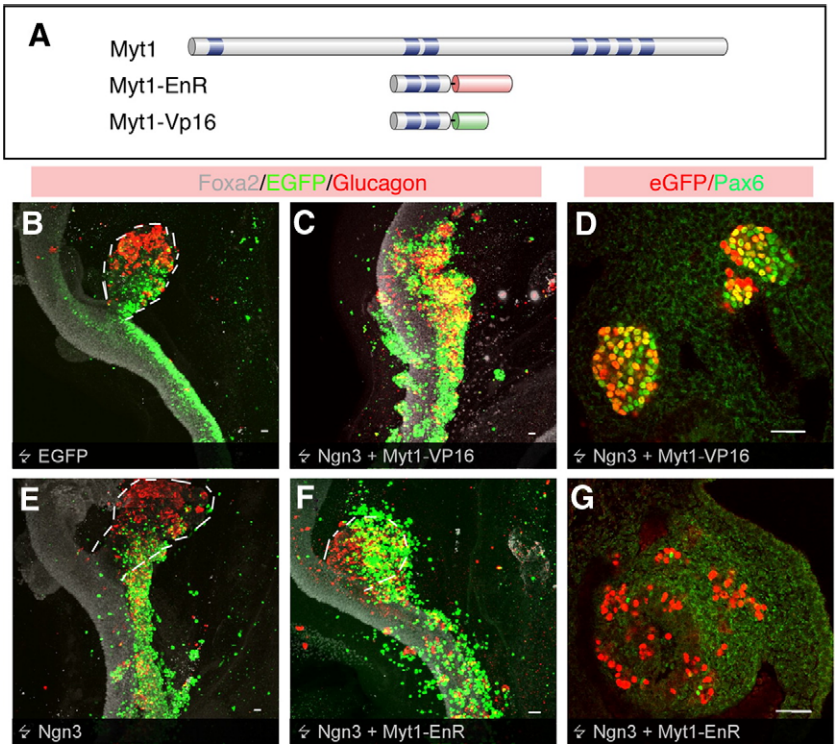


**Fig. 7.** Transactivating properties of Myt1 is required for endocrine differentiation. (A) A scheme showing the structures of Myt1–EnR and Myt1–Vp16. cDNA encoding zinc fingers 2 and 3 of Myt1b was cloned in frame with the engrailed repressive domain (Myt1–EnR) or the VP16 activation domain (Myt1–VP16) to obtain the desired open reading frames. (B–G) Endocrine marker expression in chicken endodermal cells electroporated with different DNA constructs, all shown as confocal image projections. (B) A chicken embryo electroporated with EGFP alone. Note that electroporated cells (green) could be observed in the dorsal part of the gut tube while glucagon-expressing cells (red) could only be detected in the dorsal pancreas. (C and D) Results of *Ngn3* and *Myt1–VP16* co-expression. Note the expression of glucagon (C) and *Pax6* (D) in electroporated cells (green). The endogenous pancreas in panel C is outside of the view. (E) An embryo electroporated with mouse *Ngn3* expressing plasmid alone. Note glucagon expression outside the pancreatic bud (white broken lines). (F and G) Embryos with *Ngn3* and *Myt1–EnR* co-expression. Note the delamination of electroporated cells with or without Myt1–EnR production outside the endogenous pancreas (white broken lines, F). Also note the dramatic reduction in the number of Pax6$^+$ cells in the cells that ectopically express *Ngn3* and *Myt1–EnR* (G). In panels B, C, E, and F, Foxa2 expression (grey color) highlights the gut tube. Bars = 20 μm.

and/or *Ngn3* expression. A positive feedback loop between these molecules then ensures robust Ngn3 production so that the cell could escape lateral inhibition and differentiate. This model is consistent with several other findings: (1) In *Xenopus*, *xMyT1* and *xNgnR1* (*Myt1* and *Ngn3* orthologues) co-expression allows for neuronal differentiation independent of Notch activation, whereas each molecule alone is unable to do so (Bellefroid et al., 1996); (2) In chicken embryonic endodermal cells, *Ngn3*-induced endocrine differentiation (Grapin-Botton et al., 2001) could be repressed by a constitutively active Notch molecule, NICD, suggesting that Ngn3 is not the only Notch target required for endocrine differentiation (Ahnfelt-Ronne et al., 2007a); (3) As presented in this manuscript, *Ngn3*-independent *Myt1* expression is detected in pancreatic cells. Taken together, these studies suggest that the feed-forward expression between *Myt1* and *Ngn3* plays a critical role in proper endocrine differentiation. Nonetheless, because the time frame for *Ngn3* expression within a particular cell is not clear, nor how long it takes for Ngn3 to induce *Myt1* expression, our data do not directly prove the above model. It is likely that a combined loss of Myt1/Myt1L/Myt3 will be required to directly examine whether this family of proteins is required to potentiate *Ngn3* expression during endocrine specification.

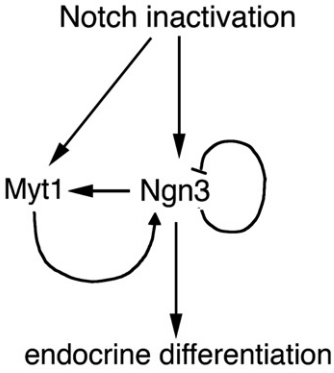


**Fig. 8.** A model for Myt1–Ngn3 interaction. Notch inactivation may induce the expression of Myt1 and Ngn3. The presence of both Myt1 and Ngn3 promotes endocrine differentiation synergistically.

Ectopic *Myt1b* expression in early pancreatic progenitors, like *Ngn3*, only results in the production of glucagon expressing cells, the cell type produced during the first wave endocrine differentiation (Apelqvist et al., 1999; Grapin-Botton et al., 2001; Schwitzgebel et al., 2000). These data suggest that Myt1b, like Ngn3, does not direct islet cell type specification when precociously expressed in early endocrine progenitors. Instead, the final identity of the endocrine cell type is likely determined by the nature, or cellular competence, of the pancreatic progenitor cells, as demonstrated recently by temporally controlled *Ngn3* activation in pancreatic progenitor cells (Johansson et al., 2007). Nonetheless, because we were unable to specifically activate *Myt1b* expression in *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/+}$ or *Myt1b*$^{tet2}$; *Pdx1*$^{tTA/tTA}$ pancreas at later embryonic stages using Dox, we could not test this hypothesis directly. It is puzzling that we could not achieve significant ectopic gene activation in later embryonic stages and in postnatal pancreas with any of the Tet-based *Myt1b* and *Ngn3* (data not shown) transgenic mouse lines. One possibility is that due to the lethality caused by the leakiness of the *Tet-Myt1b* and *tet-Ngn3* transgenes, only weak responder mice can survive to produce stable mouse lines. In this case, later pancreatic progenitors may have a lower

tTA level and unable to activate transgene expression. Alternatively, epigenetic modification of the transgene or other unknown mechanism(s) can repress the transgene expression at later embryonic stages.

While *Ngn3* ectopic expression in pancreatic progenitors directs most, if not all of the pancreatic progenitors to an endocrine fate, only a portion of the ectopic Myt1$^+$ cells switches on hormone expression. It is possible that Myt1 only accelerates the differentiation of progenitor cells that have a low level of *Ngn3* expression towards endocrine fate. These low-Ngn3-expressing cells, without the presence of *Myt1b* expression, may not become high Ngn3 expressors and thus may not undergo endocrine differentiation. It would be interesting to examine whether loss of *Myt1* function compromise *Ngn3* expression in developing pancreatic cells. Due to compensatory activation of *Myt1L* and *Myt3* in *Myt1*$^{-/-}$ pancreas, resolving this issue will likely require the simultaneous inactivation of *Myt1*, *Myt1L* and *Myt3* in the future.

One surprising finding is that a small, yet significant, number of glucagon$^+$ cells was observed in the *Ngn3*$^{-/-}$ pancreata before E15.5, after which these cells disappeared. Most of these cells expressed *Myt1*. One explanation for this observation is that these rare endocrine cells were produced in a Ngn-independent manner, where Myt1 itself is sufficient to switch on the endocrine differentiation. Alternatively, it is possible that the hormone expressing cells in the *Ngn3*$^{-/-}$ pancreas are the result of compensatory expression of *Ngn1* and/or *Ngn2*, with which Myt1b could cooperate to induce endocrine differentiation (Sommer et al., 1996). In addition, we do not know why endocrine hormone expressing cells could not be detected in *Ngn3*$^{-/-}$ pancreas in other settings, as reported by others (Gradwohl et al., 2000). One possibility is that a slight variation in the genetic background makes some animals more liable for compensatory expression of *Ngn3* paralogs. This possibility could be explored by breeding the null *Ngn3* mutation to pure genetic backgrounds in the future. Nonetheless, the detection of these endocrine cells in *Ngn3*$^{-/-}$ pancreas bolsters the notion that endocrine progenitors are heterogeneous in terms of their origin. In practice, it suggests that functional islet cells could be obtained from Ngn3-independent pathways under specific growth conditions.

Overall, this study suggests that Myt1 and Ngn3 form a feed-forward loop to enhance the expression of each other (Fig. 8). This synergistic effect could act to ensure that a sufficient number of endocrine islet cells are produced during embryogenesis. It further emphasizes the importance of introducing gene networks rather than single gene products to cells in order to obtain differentiated cells for regenerative medicine.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.02.052.

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