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Loss of *Myt1* function partially compromises endocrine islet cell differentiation and pancreatic physiological function in the mouse

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Abstract

Myelin transcription factor 1 (*Myt1*) is one of the three vertebrate C2HC-type zinc finger transcription factors that include *Myt1 (Nzf1), Myt1L (Png1),* and *Myt3 (Nzf3, St18)*. All three paralogs are widely expressed in developing neuronal cells. Yet their function for mammalian development has not been investigated directly. Here we report that only *Myt1* is expressed in the embryonic pancreas, in both endocrine progenitors and differentiated islet cells. *Myt1*$^{-/-}$ animals die postnatally, likely due to confounding effects in multiple tissues. The endocrine tissues in the embryonic *Myt1*$^{-/-}$ pancreas contained abnormal islet cells that expressed multiple hormones; although hormone levels were normal. We also created pancreas-specific *Myt1* knockout mice. These mutant animals had no obvious physical defects from their wild type littermates. Male mutant animals had reduced glucose-clearing abilities and abnormal multi-hormone-expressing cells present in their endocrine islets. In addition, they also had reduced Glut2 expression, and attenuated glucose-induced insulin secretion in the adult islets. Surprisingly, the expression of the *Myt1* paralogs, *Myt1l* and *Myt3*, was induced in the embryonic *Myt1*$^{-/-}$ pancreas. The consequences of *Myt1* inactivation in the developing pancreas could be masked by activation of its paralogs, *Myt1l* and *Myt3*. These findings suggest *Myt1* is involved in proper endocrine differentiation and function.

Keywords

endocrine islet; Myt1; endocrine progenitor; compensation; redundancy; pancreas

Introduction

The endocrine islets of Langerhans contain four major cell types (α, β, δ, and PP). β cells, which account for more than 85% of the total islet cells, produce insulin. The α, δ and PP cells, each account for about 5% of the islet mass, produce glucagon, somatostatin (SS), and pancreatic polypeptide (PP), respectively (Slack, 1995). Islets also produce ghrelin from the rarer ε cell type and a portion of the α cells (Heller et al., 2005; Prado et al., 2004; Wierup et

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al., 2004). Together, these hormones have essential roles in regulating sugar metabolism and hormone production (Gauna and van der Lely, 2005; Qader et al., 2005).

Recent gene knockout and cell lineage studies described fundamental molecular and cellular mechanisms underlying islet differentiation and function. At the molecular level, multiple signaling molecules and transcription factors are involved in endocrine differentiation and/or maintenance (Edlund, 2001; Edlund, 2002; Henseleit et al., 2005; Hill, 2005; Jensen, 2004; Kim and Hebrok, 2001; Kim and MacDonald, 2002; Polak et al., 2000; Slack, 1995; St-Onge et al., 1999; Wells and Melton, 1999). For example, *Pdx1* is required for the production of all mature pancreatic cells, although immature islet cells are present in the absence of Pdx1 (Jonsson et al., 1995; Offield et al., 1996). *Ngn3* is essential for generating all endocrine tissues (Gradwohl et al., 2000). *Arx, Nkx2.2, Nkx6, Pax4, Pax6, IAP* and several other gene products interact to produce each islet cell type (Collombat et al., 2005; Collombat et al., 2003; Gierl et al., 2006; Gradwohl et al., 2000; Mellitzer et al., 2006; Sander et al., 2000; Sosa-Pineda et al., 1997; St-Onge et al., 1997; Sussel et al., 1998). At the cellular level, endocrine differentiation occurs through two phases (Jensen et al., 2000). In the mouse before E12.5, unique glucagon or insulin producing cells are generated (Teitelman et al., 1993). Yet, these early endocrine cells are mitotically inactive and assumed to not contribute significantly to the mature islet population (Herrera, 2000; Jensen et al., 2000; Offield et al., 1996). Significant adult islet cell production starts around E13.5. These newly differentiated islet cells remain mitotically inactive until E17.5, after which they undergo mitotic divisions to account for most, if not all, islet cell expansion during normal growth (Jensen et al., 2000; Wang et al., 1994; Wang et al., 1996). For β cell production, newly differentiated cells (insulin$^+$) express the transcription factor MafB. As these cells mature, they switch off *MafB* and turn on *MafA* expression (Matsuoka et al., 2003; Nishimura et al., 2006). All endocrine islet cells are derived from Ngn3$^+$ progenitors (Gu et al., 2002). The progenitors for α and β cells do not co-express glucagon or insulin (Herrera, 2000). β cells may be derived from progenitors that also express PP at an early phase of their differentiation program (Herrera, 2000).

Vertebrates have three C2HC type zinc finger transcription factor genes, *Myt1*, *Myt1l*, and *Myt3* (Armstrong et al., 1997; Bellefroid et al., 1996; Blasie and Berg, 2000; Kim et al., 1997; Kim and Hudson, 1992; Nielsen et al., 2004; Romm et al., 2005; Wrathall et al., 1998; Yee and Yu, 1998). All three genes are highly expressed in developing neural tissues (Kim et al., 1997; Lein et al., 2007; Matsushita et al., 2002). *Myt1* gives rise to two transcripts (*Myt1a* and *Myt1b* or *Nzf2b*), differing only in their 5'-regions, using alternative transcription initiation sites (Matsushita et al., 2002). Myt1a and Myt1b have 6 or 7 zinc fingers, respectively. In all tissues examined, *Myt1b* is expressed at a much higher level than *Myt1a* (Gu et al., 2004; Matsushita et al., 2002). Only one transcript is found for either *Myt1l* or *Myt3* and each encodes a protein with 6 zinc fingers. The most conserved domains in these paralogs are the zinc fingers and the putative transcriptional regulatory domains (Bellefroid et al., 1996; Yee and Yu, 1998). In *Xenopus*, xMyt1 interacts with xNgn1 to initiate neurogenesis (Bellefroid et al., 1996). In rat cell culture, Myt1 interacts with Sin3b to regulate gene expression (Nielsen et al., 2004). How Myt1 regulates specific target genes remains obscure.

We discovered *Myt1* as a gene highly expressed in a subset of developing pancreatic cells (Gu et al., 2004), possibly the endocrine progenitors that express *Ngn3* (Gu et al., 2002), through an unbiased screen for transcripts enriched in endocrine progenitors. *Myt1* mRNA was readily detected in the endocrine progenitors and loss of *Nkx6.1/Nkx6.2* function attenuated its expression (Henseleit et al., 2005). Both *Myt1a* and *Myt1b* transcripts were detected in the embryonic pancreas, with *Myt1b* being the dominantly expressed isoform (Gu et al., 2004). Ectopic *Myt1* expression in the developing chicken endodermal epithelium induced glucagon and somatostatin expression. A dominant-negative Myt1 molecule inhibited endocrine differentiation in both mouse pancreas and chicken hindgut cells (Gu et al., 2004). A Notch

modifier that induced endocrine differentiation in endodermal cells also induced *Myt1* expression (Xu et al., 2006). It was also shown that Myt1 partially antagonizes Notch activity facilitating neuro-endocrine differentiation (Ahnfelt-Ronne et al., 2007). These results suggest, but do not prove, that *Myt1* is involved in endocrine islet production and function.

We systematically analyzed *Myt1* protein expression in the pancreatic lineages and derived both global and pancreas-specific *Myt1* knockout mice. We detected Myt1 in both endocrine progenitors and in differentiated islet cells. The pancreas of *Myt1* homozygous mutant mice contained islet cells that co-expressed multiple hormones. Pancreas-specific *Myt1* knockout male mice had reduced glucose-clearing ability, islet *Glut2* expression, and glucose-induced-insulin-secretion (GSIS). Interestingly, during embryogenesis, the expression of *Myt1l* and *Myt3* were induced in the pancreas of *Myt1*$^{-/-}$ mutant mice. These data suggest that *Myt1* is involved in both endocrine differentiation and function, and losing *Myt1* function can be partially compensated by the activation of its paralogs (*i.e., Myt1I and Myt3).*

Results

Myt1 is expressed in both endocrine progenitor and differentiated endocrine cells

Our previous mRNA-based studies suggest *Myt1* is expressed in endocrine progenitors and mature islet cells (Gu et al., 2004). To determine which pancreatic cell type produces Myt1, we developed antibodies for Myt1 expression analyses (Materials and Methods).

In the developing pancreas, we detected robust *Myt1* expression after E9, but not before E8.5 (Fig. 1A1). At this developmental stage, most Myt1^{+} cells also expressed *Pdx1* (Fig. 1A), suggesting their pancreatic origin. We analyzed Myt1 production in the β cell lineage. At E10.5 when insulin^{+} cells appear, we detected Myt1 expression in all insulin^{+} cells (Fig. 1A2 and table 1). After E10.5, Myt1 expression was maintained in a portion of the insulin producing cells. Notably, the percent of insulin^{+} cells expressing Myt1 decreased with age. For example, 91% of insulin^{+} cells were Myt1^{+} at E13.5, yet only 58.6% of insulin^{+} cells maintained detectable Myt1 production at 12 weeks-of-age (Table 1). Myt1 expression is also detected in the glucagon, SS, and PP-expressing cells. Yet the percentage of these hormone^{+} cells that co-express Myt1 remains relatively constant at all the stages studied (Fig. 1B-D). Specifically, from the appearance of these hormones to three month of age, about 95% hormone^{+} cells co-express Myt1 (Table 1). These data suggest that the mature β cells are a heterogeneous population, whereas the other endocrine cell types are relatively uniform.

In the mouse pancreas, robust Ngn3 expression is detectable at E10.5, peaks at E15.5, and then is undetectable after birth. In all stages, Ngn3 is not detectable in hormone-expressing cells, demonstrating that Ngn3 can be used as an endocrine progenitor marker (Jensen et al., 2000; Schwitzgebel et al., 2000). We utilized two anti-Ngn3 antibodies, a monoclonal mouse anti-Ngn3 and a guinea pig anti-Ngn3 (Seymour et al., 2007), to examine whether *Myt1* is expressed in Ngn3^{+} cells. Both antibodies showed *Myt1* expression partially overlapped with *Ngn3* (Fig. 2). We found significantly more pancreatic cells expressing *Myt1* than *Ngn3* at all stages examined (E10.5, E13.5, and E15.5; Table 2). These data suggest that a portion of the *Myt1*-expressing cells are endocrine progenitors. In this case, *Myt1* expression could be turned on in cells that transiently express *Ngn3.* Yet *Myt1* expression is maintained in these same cells, even after *Ngn3* expression is switched off. This result is further supported by the findings that *Myt1* expression overlaps with expression of *Isl1, MafA, MafB,* and *Pax6,* which label different endocrine progenitor or islet cell types (Supplementary Fig. 1).

Myt1 is the only paralog expressed in the developing pancreas

The detection of Myt1 in both endocrine progenitor and differentiated islet cells suggests that Myt1 might participate in endocrine differentiation/function. To determine whether *Myt1* is the only paralog involved in endocrine islet development, we examined *Myt1l* and *Myt3* expression by *in situ* hybridization in E13.5, E14.5, and E15.5 pancreata. Although *Myt1l* and *Myt3* were detected in some cells within mesenchymal tissues that surround the gut epithelium [which were probably neuronal cells (Kim et al., 1997), Fig. 3 D, E and data not shown], their expression in the developing pancreas was not detectable (Fig. 3 and data not shown), suggesting that *Myt1* is the only family member likely involved in mouse pancreatic development.

Loss of *Myt1* function results in abnormal endocrine differentiation

We derived a conditional *Myt1* knock-out allele ($Myt1^{fl}$) to directly examine the role of *Myt1* in islet cell development and function. We flanked the 91 base-pair exon common to both *Myt1* transcripts (#3 exon of *Myt1a* or #9 exon in *Myt1b*, Fig. 4A-C) with tandem LoxP sites to simultaneously inactivate *Myt1a* and *Myt1b* by Cre-mediated recombination. The mutant allele introduces a frame shift that should produce truncated Myt1a and Myt1b peptides that either have no similarity to any known protein or with a single zinc finger, respectively.

Sox2-*Cre* animals (Hayashi et al., 2002) were used to delete the floxed exon, generating the loss-of-function *Myt1* allele ($Myt1^{-}$) in the germ line. Homozygous mutant animals displayed no visible abnormality yet died immediately after birth. The diaphragm of $Myt1^{-/-}$ mutant mice was not properly innervated (Supplementary Fig. 2); these mutants are likely to have died due to failure to begin breathing. Because these diaphragm-related defects could be readily scored, we determined whether the $Myt1^{-}$ allele had a dominant negative effect: if the mutant Myt1 products had a dominant negative effect, we would expect to find abnormal diaphragm innervation or reduced viability in heterozygous animals. $Myt1^{+/-}$ diaphragms were innervated similar to wild type animals (Supplementary Fig. 2). To date, we obtained 156 wild type and 329 heterozygous adult animals but no homozygous animals survived beyond birth. This Mendelian ratio demonstrates one mutant allele does not affect viability. We further analyzed the *Myt1* message levels in the wild type, $Myt1^{+/-}$, and $Myt1^{-/-}$ pancreata using *in situ* hybridization and semi-quantitative RT-PCR. The cRNA probe, derived from the full-length *Myt1* cDNA (see experimental procedures), recognized both wild type and mutant *Myt1* mRNA. Results show that mutant *Myt1* mRNA level is reduced by more than 4 fold (Fig. 4D, E, and F), making $Myt1^{-}$ unlikely to be a dominant negative allele.

We examined the expression of insulin, glucagon, SS, and PP in $Myt1^{-/-}$ pancreata at E10.5, E13.5, E15.5, and E18.5. Surprisingly, the expression levels of these hormones were not significantly different between wild type and homozygous mutant littermates, as assayed by the number of hormone^{+} cells, the relative fluorescence intensity within each hormone-expressing cells, or the total hormone (insulin and glucagon) content within each pancreas (Supplementary Fig. 3 and data not shown). Consistent with these findings, the number of Ngn3^{+} endocrine progenitors, as well as the Ngn3-expression levels within individual cells in wild-type, heterozygous, and homozygous mutant embryos at E10.5, E13.5, and E15.5 did not vary neither (Supplementary Fig. 4).

When assayed together, the mutant endocrine compartment contained a substantial percentage of cells that co-expressed insulin and PP, or SS and PP, at all stages, including E15.5 and E18.5 (Fig. 5A-D), whereas other hormone co-expression combinations were not found at significant numbers (Fig. 5E, F, and data not shown). At E15.5, when PP^{+} cells became detectable, less than 1.5% PP^{+} cells expressed detectable insulin in wild type and $Myt1^{+/-}$ pancreata. Yet 11.6 ±2.4% (mean ± standard deviation) of PP^{+} cells expressed insulin in $Myt1^{-/-}$ pancreata.

Similarly, E15.5 *Myt1*$^{-/-}$ pancreata contained 19.9±3.1% PP^{+} cells that expressed SS, whereas less than 2% PP^{+} cells expressed SS in wild type and *Myt1*$^{+/-}$ pancreata (Fig. 5). At E18.5, 7.8±2.1% or 21.6±4.2% PP^{+} cells also expressed insulin or SS, respectively. In wild type or *Myt1*$^{+/-}$ pancreas, less than 3% of PP^{+} cells expressed either insulin or SS. Student T-test showed that the presence of these double-hormone^{+} cells in the homozygous mutant pancreas was statistically significant ($p<0.01$). At all embryonic stages, we could not detect significant number of cells that co-express glucagon and insulin (Fig. 5E and F). These findings demonstrate that *Myt1* is required for proper endocrine differentiation or for differentiated cells to maintain specific hormone production.

Adult *Myt1*$^{fl/-}$; *Pdx1-Cre* pancreas has immature endocrine cells

To determine whether loss of *Myt1* function compromises endocrine islet function, we derived pancreas-specific *Myt1* nullizygous animals using *Pdx1-Cre* transgenic mice (*Myt1*$^{fl/-}$*; Pdx1-Cre*), which inactivated *Myt1* only in the *Pdx1*-expressing pancreatic, duodenal, and stomach cells (Gu et al., 2002). We examined the efficiency of *Myt1* deletion in these embryos and found that by E10.5 or E13.5 (n=2), Myt1 expression in the pancreas was reduced by 60% or 88% over that of wild type littermates, respectively. By E18.5, we did not detect Myt1 expression in *Myt1*$^{fl/-}$*; Pdx1-Cre* pancreatic cells [(n=2), Supplementary Fig. 5]. Consistent with the findings in the global *Myt1*$^{-/-}$ pancreas, E15.5 and E18.5 *Myt1*$^{fl/-}$*; Pdx1-Cre* pancreata contained significant numbers of insulin^{+}-PP^{+} and PP^{+}-SS^{+} cells, but not glucagon^{+}-insulin^{+} cells (Fig. 5E and F).

Both male and female *Myt1*$^{fl/-}$*; Pdx1-Cre* animals were viable, fertile, and indistinguishable in weight from their littermate controls. Islet morphology remained unchanged between homozygous mutant and wild type littermates. Yet the adult *Myt1*$^{fl/-}$*; Pdx1-Cre* islet compartments (12 weeks of age) contained a significant portion of PP^{+} cells expressing either insulin or SS (Fig. 6). Specifically, wild type islets contained 2.4±0.6% PP^{+} cells that expressed SS (n=3), whereas *Myt1*$^{fl/-}$*; Pdx1-Cre* islets contained 10.2±5.4% PP^{+} cells that expressed SS (n=3). Similarly, wild type islets contained 0.2±0.1% PP^{+} cells that expressed insulin (n=3), whereas *Myt1*$^{fl/-}$*; Pdx1-Cre* islets contained 4.1±0.6% PP^{+} cells that expressed insulin (n=3). Student T-test demonstrated that the presence of these double-hormone^{+} cells in the *Myt1*$^{fl/-}$*; Pdx1-Cre* pancreas was significantly different from that of wild type pancreas ($p<0.01$).

In addition to insulin^{+}- PP^{+} cells and PP^{+}- SS^{+} cells, we observed a significant number of glucagon^{+}- insulin^{+} cells, accounting for 2.7±0.8% (n=3) of total glucagon^{+} cells, appeared in *Myt1*$^{fl/-}$*; Pdx1-Cre* mutant islets. These double hormone^{+} cells were distributed in 8% of the islet sections. In wild type littermates, 0.3±0.1% (n=3) of glucagon^{+} cells expressed detectable insulin (Fig. 6), consistent with previous findings that double hormone^{+} cells were rarely found in wild type pancreas (Guz et al., 1995;Herrera, 2000).

MafA and MafB expression reflects the maturity of pancreatic α and β cells (Nishimura et al., 2006). If double hormone-expressing cells were immature, they might express an abnormal pattern of *MafA* and *MafB*. Indeed, MafA was detected in most wild type β cells, but not in any of the insulin^{+}PP^{+} and glucagon^{+}insulin^{+} cells examined (Fig. 6 A and B). However, most abnormal insulin^{+} cells (19/21 insulin^{+}PP^{+} and 26/26 glucagon^{+}insulin^{+} cells) in the mutant pancreas maintained MafB production (Fig. 6C and D). These findings demonstrate these double hormone positive cells, even though they express insulin, are not mature β cells.

Adult *Myt1*$^{fl/-}$; *Pdx1-Cre* males have glucose intolerance and insulin secretion abnormality

To investigate whether *Myt1* inactivation compromises endocrine function, we examined the blood glucose levels in the adult pancreas-specific mutant animals. The *Myt1*$^{fl/-}$*; Pdx1-Cre*

mutant and wild type mice showed no difference in their fasting glucose level (data not shown). Yet glucose tolerance test (GTT), a more sensitive assay for general pancreas homeostatic function, revealed that *Myt1*$^{fl/-}$*; Pdx1-Cre* male mice developed glucose intolerance when they were older than 6 weeks of age (Fig. 7A and data not shown). Consistent with this phenotype, 10-weeks old *Myt1*$^{fl/-}$*; Pdx1-Cre* males displayed an attenuated glucose-induced insulin secretion: 30 minutes after glucose injection, wild-type animals showed a 100% serum insulin level increase while the mutant animals had only a 30% increase (Fig. 7B). This result is consistent with the findings that *Myt1*$^{fl/-}$*; Pdx1-Cre* mutant islets had reduced Glut2 protein levels (Fig. 7C). The serum glucagon between wild type and mutant animals, before and after glucose challenge, had no significant alterations (data not shown). These results suggest that reduced insulin secretion was likely responsible for glucose intolerance.

Finally, we examined the glucose-stimulated insulin secretion (GSIS) from *Myt1*$^{fl/-}$*; Pdx1-Cre* male islets. Islets isolated from 5 weeks old mutant males, when no glucose intolerance could be detected, displayed no GSIS defects (data not shown). 7 weeks old mutant islets had no statistically significant reduction in GSIS as well. As expected, the GSIS defect in isolated *Myt1*$^{-}$ nullizygous islets became more prominent as the males aged. At 12 weeks of age, the mutant islets had statistically significant reduction in their GSIS (Fig. 7D). These data suggest that *Myt1* is required for the development of functional islet cells.

Myt1l and *Myt3* are expressed in the *Myt1*$^{-/-}$ mutant pancreas

Our previous analyses using a dominant negative Myt1 molecule hinted that *Myt1* is essential for endocrine differentiation (Gu et al., 2004). Because neither *Myt1l* nor *Myt3* mRNA were detected in the wild type embryonic pancreas, we were puzzled by the weak endocrine phenotype in the *Myt1* homozygous mutants. We examined whether *Myt1l* or *Myt3* expression was induced in the *Myt1* mutant pancreas, potentially compensating for the loss of *Myt1* function. We found substantial levels of both *Myt1l* and *Myt3* mRNAs in the embryonic pancreas in *Myt1* global and pancreas-specific mutant pancreata at E13.5, E14.5, and E15.5 (Fig. 8 and data not shown). Because *Myt1, Myt1l* and *Myt3* have highly similar zinc fingers and structural domains (Yee and Yu, 1998), this finding suggests that loss of *Myt1* function may be compensated by *Myt1l* and/or *Myt3* activity.

Discussion

Endocrine islet differentiation is a multiple-step process requiring proper expression of many factors (Collombat et al., 2006; Edlund, 2001; Edlund, 2002; Henseleit et al., 2005; Hill, 2005; Jensen, 2004; Kim and Hebrok, 2001; Kim and MacDonald, 2002; Polak et al., 2000; Slack, 1995; St-Onge et al., 1999; Wells and Melton, 1999). Although loss-of-function analyses of these genes provide a general picture on how these factors control the production of various islet cell types at the molecular level, data are less clear on how the early progenitors progress step-by-step to mature, functional specific hormone-expressing islet cells. Our data suggest Myt1 is involved in multiple steps of endocrine differentiation and function.

Myt1 was detected in both hormone^{+} and hormone^{-} cells during early embryogenesis, suggesting Myt1 might participate in both endocrine differentiation and function. Many Myt1^{+} cells at E9 expressed Pdx1, but not endocrine hormones. In later stages, Myt1 was detected in Ngn3^{+} endocrine progenitors and hormone-expressing cells. These findings suggest the early Myt1^{+} cells are derived from Pdx1^{+} progenitor cells and they differentiate to endocrine cells. The fact that *Myt1* expression was maintained in most, but not all islet cells, proposes that Myt1 might help islet cells maintain their proper identities. The appearance of glucagon^{+}insulin^{+} cells in the adult *Myt1*$^{-/-}$ mutant islets, but not in the late *Myt1*$^{-/-}$ embryonic islets is also consistent with this hypothesis.

The reasons why insulin$^+$/PP$^+$ and PP$^+$/SS$^+$ cells are found in *Myt1* mutant embryonic pancreas are not known. A transient PP$^+$ endocrine precursor pool exists in the embryonic stages. These PP$^+$ cells could give rise to insulin and/or SS-producing cells in the mature islets. Yet the insulin$^+$/PP$^+$ and PP$^+$/SS$^+$ cells could not be detected in the wild-type pancreas because they might quickly turn off PP expression as they become β and/or δ cells. Loss of *Myt1* function might slow down this process and allow detection of these intermediate cells. Existing data support this possibility. At least a portion of the adult β cells arise from a PP-expressing progenitor population (Herrera, 2000). Whether the SS-expressing δ cells could also come from these precursors was not reported. A second possibility of why insulin$^+$/PP$^+$ and PP$^+$/SS$^+$ cells exist in *Myt1*$^{-/-}$mutant embryos is that Myt1 is required during the acquisition or maturation of the β or δ cell fate and to repress PP cell fate. For example, loss of *Pax4* function abolishes β and δ cell production, yet boosts α cell generation (Sosa-Pineda et al., 1997). Loss of *Arx* function results in ablation of α cells with concomitant increase in β and δ cells (Collombat et al., 2005; Collombat et al., 2003). In these reports, cells that co-express more than one hormone were not reported. A third possibility is that *Myt1* is involved in PP cell differentiation. In this case, loss of *Myt1* function results in failure of PP cell maturation and leaky expression of other endocrine hormones. A temporally controlled PP cell marking experiment would provide clarity.

Myt1$^{fl/-}$*; Pdx1-Cre* adult males have glucose intolerance. This phenotype is consistent with the presence of abnormal islet cells, reduced Glut2 expression, and reduced GSIS in the mutant islets. The number of abnormal insulin$^+$ cells represents only a small fraction of β cells. A small number of abnormal endocrine cells might dominantly interfere with β cell communication and result in glucose intolerance (Konstantinova et al., 2007). Alternatively, Myt1 could be required for Glut2 expression and maintenance in mature β cells. Thus, inactivating *Myt1* results in the gradual loss of Glut2 in the mutant islets and subsequent glucose intolerance.

The weak pancreatic phenotype in the *Myt1* mutants could be due to the compensation of *Myt1l* and *Myt3*. These paralogs have highly conserved DNA binding and transcription regulatory domains (Kim et al., 1997). Although the expression of these two genes were not detected in the wild type embryonic pancreas, high levels of *Myt1l* and *Myt3* messages were present in *Myt1*$^{-/-}$ mutant pancreas. A triple mutant, including *Myt1*, *Myt1l* and *Myt3*, is likely required to fully decipher the role of these transcriptional regulators in endocrine differentiation, maintenance, and function. Lastly, how the Myt1 protein level in the embryonic pancreas regulates the expression of *Myt1l* and *Myt3* is a subject requiring further investigation. Results from these further studies could potentially reveal a feedback compensation mechanism that allows for robust endocrine tissue regeneration under physiological stress, and therefore provides another venue for regeneration-based diabetes therapy.

Experimental procedures

Mouse strains and care

Mouse husbandry and genotyping were performed following standard protocols. Mouse care followed protocols (M/03/363 and M/03/354) approved by the Vanderbilt Medical Center IACUC. For embryonic staging, 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 initial knockout mice production, C57BL/6 strain was used (Charles River Laboratories, Inc. Wilmington, MA). Subsequent strain maintenance and crosses utilized CD1 mice. *Flpe* (used to delete the selection marker in the targeted allele), *Pdx1-Cre* and *Sox2-Cre* mice were previously reported (Dymecki, 1996; Gu et al., 2002; Hayashi et al., 2002).

Generation of Myt1 antibodies

To produce Myt1 antibodies, an open reading frame encoding Myt1 amino acid residues 109-298 (as numbered in mKIAA0835) was fused with that encoding a maltose binding protein (New England Biolabs, Beverly, MA). The fusion protein was purified and used as an antigen. This portion of the Myt1 peptide will be deleted in the mutant *Myt1* allele. Antibody production in rabbits was performed by Strategic Bio-solutions (Newark, DE). The specificity of the antibodies was shown by the lack of positive signals in *Myt1*$^{-/-}$ mouse tissues (Supplementary Fig. 5).

In situ hybridization and immunohistochemistry/immunofluorescence

In situ hybridization and immunostains followed established protocols. Briefly, mouse tissues were fixed in 4% paraformaldehyde overnight at 4°C or 4 hours at room temperature. For *in situ* hybridization, tissues were prepared as 6 μm paraffin sections. All cRNA probes were made using cDNA clones as templates. The *Myt1* cDNA (#LDN 147) was a gift from L. Hudson (Kim et al., 1997). This cDNA clone was digested with XhoI and transcribed with T7 RNA polymerase. The *Myt1l* (#6844118) and *Myt3* (#5366688) cDNA clones were purchased from Open Biosystem (Huntsville, AL). These cDNA clones were digested with Sal I or Avr II and transcribed with T3 or T7 RNA polymerase for cRNA probe production, respectively. For immunostaining, either frozen or paraffin sections were utilized. Primary antibodies used: guinea pig anti-insulin, goat anti-c-peptide (recognizing the same cells as anti-insulin antibodies), and guinea pig anti-glucagon (Dako, Carpinteria, CA), rabbit anti-glucagon, rabbit anti-SS, goat anti-SS and guinea pig anti-PP (Invitrogen, Carlsbad, CA). Rabbit anti-Glut2 (Chemicon, Temetula, CA). Rabbit anti-MafA and MafB, gifts from R. Stein (Matsuoka et al., 2004). Goat anti-Pdx1, a gift from C. Wright. Mouse anti-Ngn3 (F25A1B3), Hybridoma Bank, Iowa, IA. Guinea pig anti-Ngn3, a gift from M. Sander (Seymour et al., 2007). Secondary antibodies used: 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 (Jackson Immunoresearch, West Grove, PA). Cy5-conjugated donkey anti-rabbit IgG, Cy5-conjugated donkey anti-guinea pig IgG (Chemicon, Temetula, CA). For all antibodies, a1:500-1:2000 dilution was used, depending on the amount of tissue on each slide.

Myt1 targeting constructs and animal derivation

The targeting vector pGKNeo-DTA was a gift from A. McMahon (Zhang et al., 2001). This vector contains a pBluescript KSII backbone, a diphtheria toxin A subunit (DTA) as negative selection marker, and a FRT-flanked pGKneo gene as a positive selection marker (Zhang et al., 2001). A genomic fragment flanking the third exon of *Myt1a* was PCR-amplified, sequenced, and cloned into the Fse I site of the vector. The primers used were: forward: 5'-aggccggccaaagattaaagtttagag-3' and reverse: 5'-aggccggccctaccatgccccagcttaat-3'. The template was BAC #519N6 in library RPCI22, purchased from Children's Hospital of Oakland, Oakland, CA (Warming et al., 2005). Another 3.5 kb genomic DNA fragment 3' of the deleted region was PCR-amplified, sequenced, and cloned between the Sal I-Pme I sites of the vector. The primers used were: forward, 5'-agtttaaacgaaattagggctgggcctgt-3'; reverse: 5'-agtcgacgcagttttaaaatcaccctcag-3'. Finally, an 8.2 kb genomic fragment 5' of the deleted region, derived through BAC-based recombineering, was ligated to the Not I-Asc I site to complete the targeting vector construction. The oligos used for BAC recombineering were: 5'-ggcgcgccctcgagtgctgagactacaggtatgcaccaccatgctctgctaaggatagaatccctccagagagtgagta gcttcatgtagggttatcccgcggccgc-3' and its complimentary strand. Oligos used to amplify 5' probe were: forward: 5'-atattaggaatattaaaacttgt-3'. Reverse: 5'-ggtcacaatgatgggcactaac-3'. Oligos for the 3' probe: forward: 5'-ctacataaaccttcaaggtc-3'. Reverse: 5'-gaattcaattcccagcaaccaca-3'. Targeted ES cells were utilized to derive chimera mouse following standard procedure. After

mouse line establishment, *Flpe* mice were used to delete the FRT-flanked pGK-Neo selection cassette (Dymecki, 1996), deriving the *Myt1*^{fl} allele. Oligos for PCR genotyping: Pr1: 5'-agatccttccagggtggagaagc-3'; Pr2: 5'-gtctgtccagaacctattccaga-3'; Pr3: 5'-cagacttccattcccacagtt-3'. Expected DNA fragment length for the wild type, the floxed (*Myt1*^{fl}), and the deletion allele (*Myt1*$^{-}$) is 490, 380, and 340 bps, respectively. Oligos for assessing the stability of mutant *Myt1* messages: 5'-gagctatctagtcctaaacctga-3' and 5'-ttgggaggatctcctgtctgcaa -3'. Expected length of wild type fragment: 608bp, mutant fragment: 517 bp. After RT-PCR, the intensities of the bands were quantified using BIOQUANT true-color windows system (R & M Biometrics, Nashville, TN). Gene targeting was performed using TL1 ES cells and three correctly targeted clones were identified from 219 colonies screened. Blastocyst microinjections were performed by the Vanderbilt Transgenic/ES Cell Shared Resource, following standard procedures.

Glucose tolerance test and *in vivo* insulin secretion assay

GTT followed published procedure (Lammert et al., 2003). Briefly, after overnight fasting (16 hours), mice were injected intraperitoneally with glucose at 2g per kg body weight. Blood glucose was monitored with a compact glucose analyzer (BD /Medtronic MiniMed Blood Glucose Monitor) at different time points after glucose administration. For insulin secretion assay, blood samples were taken before or 30 minutes after glucose injection. Serum insulin levels were assayed using the Elisa assay kit from Linco following manufacturer-recommended protocol (St Charles, MO).

Islet isolation and glucose stimulated insulin secretion assay (GSIS)

Pancreatic islet isolation was by collagenase perfusion (Gu et al., 2004). Batches of 10-15 hand-picked islets of similar size were incubated in RPMI 1640-10% fetal calf serum at 37°C for overnight. These islets were washed and incubated in 1 ml Krebs-Ringer HEPES-buffered saline in 2.8 mM glucose at 37°C for 30 min and then transferred to 1ml Krebs-Ringer HEPES-buffered saline in 20 mM glucose for another 30 min. Supernatants were collected for insulin assay. Islets were extracted for total insulin assay. Insulin secretion was calculated as percent of insulin released within the 30 min glucose stimulation. All assays utilized triplicate samples.

Confocal microscopy for protein expression and statistical analysis

All fluorescent images in the figures were from confocal microscopy. Typically, optical sections at 0.4-0.6 μm intervals were taken. A single or a projection of two adjacent optical sections was used for a high quality image. All confocal parameters were kept consistent throughout our experiments. For quantification, we utilized BIOQUANT true-color windows system (R & M Biometrics, Nashville, TN). This system assays both cell number and fluorescence intensities (Zhang et al., 1997). The hormone amount quantified using this approach matched well with our Elisa-based total insulin or glucagon assay data from E18.5 embryonic pancreas.

For Myt1 staining, 10-15 μm frozen sections were used. For Glut2 expression or co-hormone expression analysis, 4-8 μm paraffin sections were used. For co-hormone expressing cell quantification at E15.5 and E18.5, adjacent 10μm frozen sections were separated onto groups of three slides, with one staining for glucagon/insulin, one for insulin/PP, and one for PP/SS. Co-stained cells were counted on optical sections at 6 μm apart. Two optical sections from each tissue section were counted, and all tissue sections from each E15.5 pancreas were analyzed. A third of all tissues from each E18.5 pancreas were counted (to ensure correct representation of the samples, three adjacent sections were collected onto three individual slides. Then the next six sections were skipped before further tissue collection). For quantification in adults, each pancreas was cut into 25-30 small pieces and embedded into a single paraffin block. One of every four 4μm sections was collected, with sets of three being

mounted onto groups of three slides for glucagon/insulin, insulin/PP, and SS/PP staining respectively. Slides were then counterstained with DAPI and co-stained cells were scored using a Carl Zeiss Axioplan 2 fluorescence microscope. At least 25 sections were scored for each hormone combination in each pancreas. Statistical analyses utilized standard student T-test. A p-value of 0.05 or better was considered significant. All quantification data were presented as (mean ± standard deviation).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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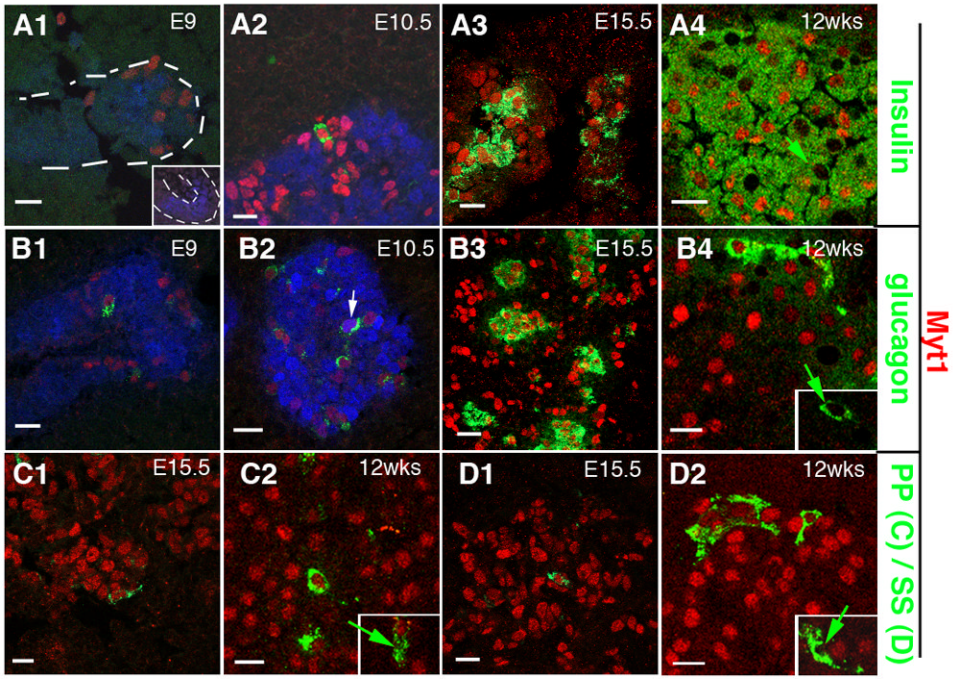

Figure 1. Myt1 is detected in endocrine progenitor and differentiated islet cells

Red fluorescence in all panels represents staining against Myt1. Blue, Pdx1 staining (only A1-2, B1-2 samples were stained for Pdx1). Green: A1-4, insulin; B1-B4, glucagon; C1-2: PP; D1-2: SS. Inset in A1 is an E8.5 pancreatic epithelial section stained for Myt1, insulin, and Pdx1. White broken lines outline the prospective pancreatic epithelium. Insets in B4, C2, and D2 show examples of hormone^{+}Myt1^{-} cells. Green arrows in A4, B4, C2, and D2 point to Myt1^{-}hormone^{+} cells. White arrow in B2 points to a glucagon^{+}Myt1^{+}Pdx1^{+} cell. Scale bars=20 μm.

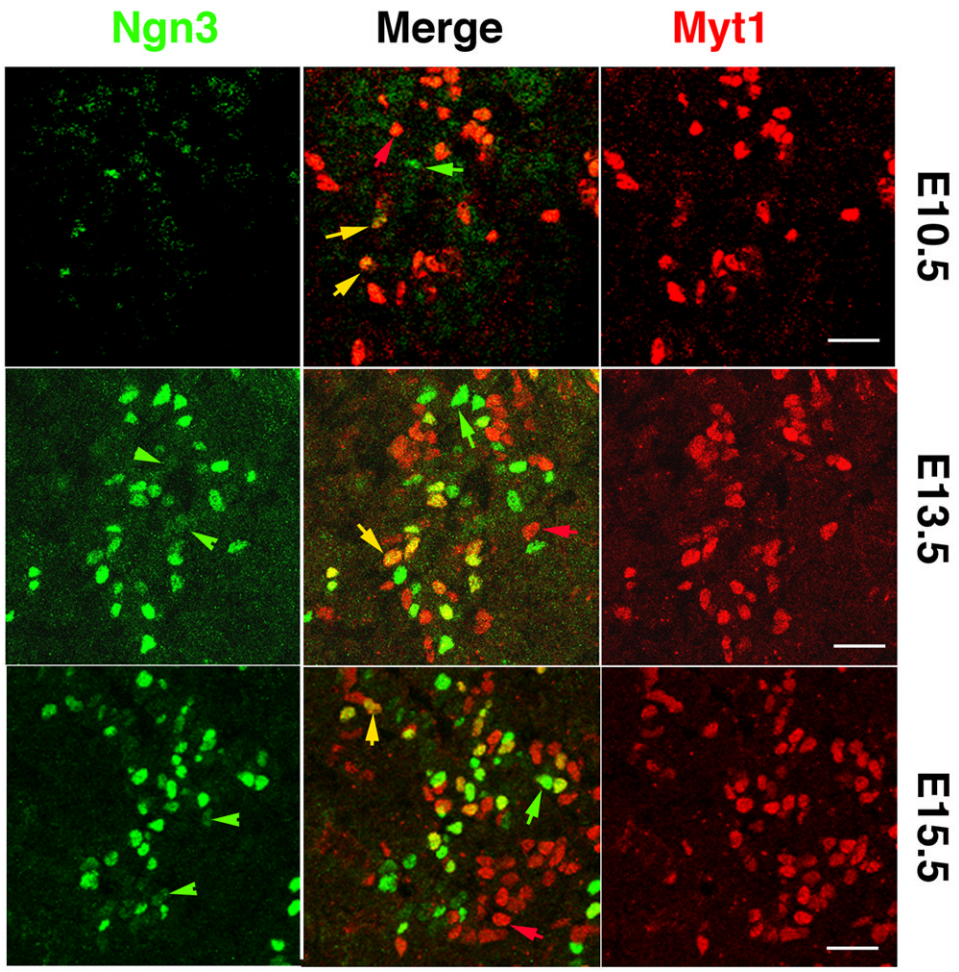

Figure 2. *Myt1* expression overlaps with that of *Ngn3*

Shown were Myt1 and Ngn3 co-staining in E10.5, E13.5, and E15.5 pancreatic cells, respectively. The middle column is a merge between the left and right columns. The double positive cells display a yellow color as a result of overlapping red and green fluorescence. Yellow arrows: $Myt1^{+}Ngn3^{+}$ cells. Green arrows: $Myt1^{-}Ngn3^{+}$ cells. Red arrows: $Myt1^{+}Ngn3^{-}$ cells. Green arrowheads point to some low-Ngn3-producing cells. Scale bars=20μm.

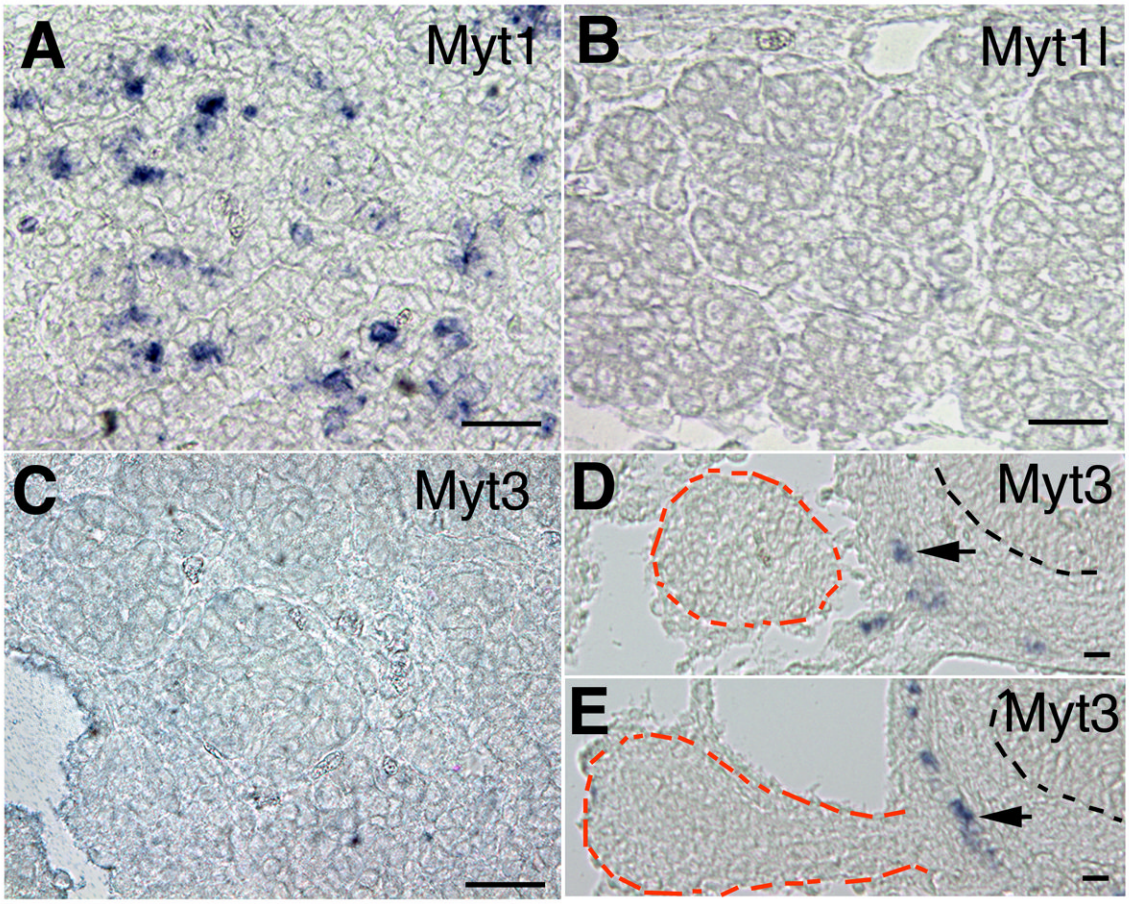

Figure 3. *Myt1* is the only paralog expressed in developing mouse pancreas

The *Myt1*, *Myt1l*, and *Myt3* expression in embryonic pancreas as detected by *in situ* hybridization. In panels A, B, and C, only pancreatic regions are shown. Color development in A is 24 hours (see Materials and Methods). Color development in B and C is 72 hours. (D, E), positive controls showed *Myt3* expression in cells that surround the duodenal epithelium (black broken lines), but not in cells within pancreatic buds (red broken lines). Tissues used in A, B, and C are of E14.5. Tissues used in D and E are of E13.5. Scale bars=20μm.

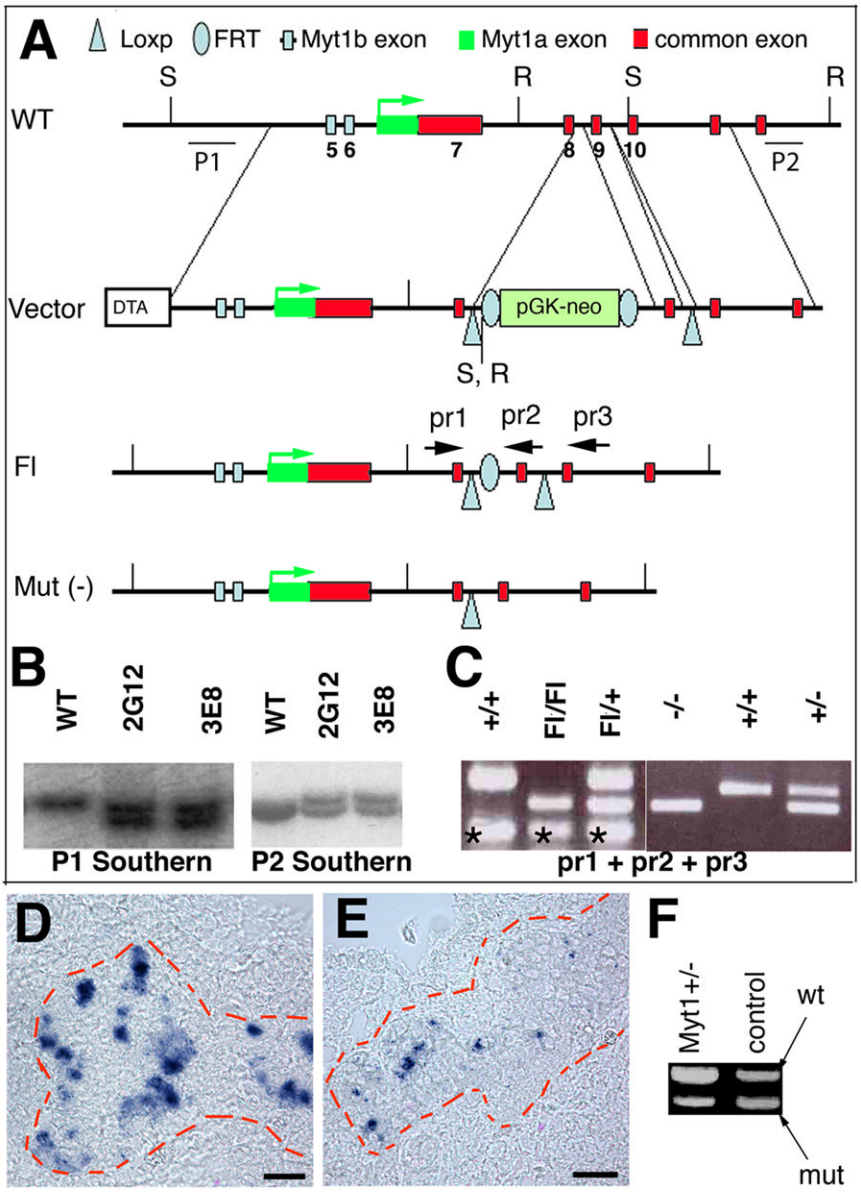

Figure 4. *Myt1* locus targeting strategy results in decreased mutant *Myt1* mRNA accumulation in *Myt1*$^{-/-}$ pancreas

(A), targeting vector. Final targeted allele (Fl) has two LoxP sites flanking exon 3 of *Myt1a* (or exon 9 of *Myt1b*). Note the position of the DNA fragments, P1 and P2, for DNA Southern blot probes and the oligos (Pr1, Pr2, and Pr3) for genotyping. (B), Southern blots of two targeted ES cell clones, 2G12 and 3E8. Spe I (S) digestion and blotting with P1 probe produced a wild-type band of 14.5 kb and a targeted band of 12 kb. EcoR I (R) digestion and blotting with P2 probe resulted in a wild-type band of 6 kb and a targeted band of 7 kb. (C), PCR-based genotyping using DNA oligos pr1, pr2, and pr3. The bands marked with "*" were primer dimmers. (D and E), *in situ* detection of *Myt1* mRNA in both wild type (D) and *Myt1*$^{-/-}$ (E) pancreata at E13.5. cRNA was generated from a full length *Myt1* cDNA, which also recognized the *Myt1* mutant mRNA. Red broken lines circled the pancreatic epithelial region. (F), semi-quantitative RT-PCR results from *Myt1*$^{+/-}$ pancreas. The higher or lower band is the wild type or mutant product band, respectively. Left lane: RT products of *Myt1*$^{+/-}$pancreas as template. A comparison of the intensity of the two bands (1:3.1 ratio as determined by BIOQUNT)

reveals relative abundance of the mutant and wild type mRNA. Right lane, a mix of expected PCR products at 1:1 molecular ratio as a control template, to show the amplification efficiency of the two fragments. The lower band has a higher PCR amplification efficiency for the shorter fragment (1:1.3). Scale bars=20 μm.

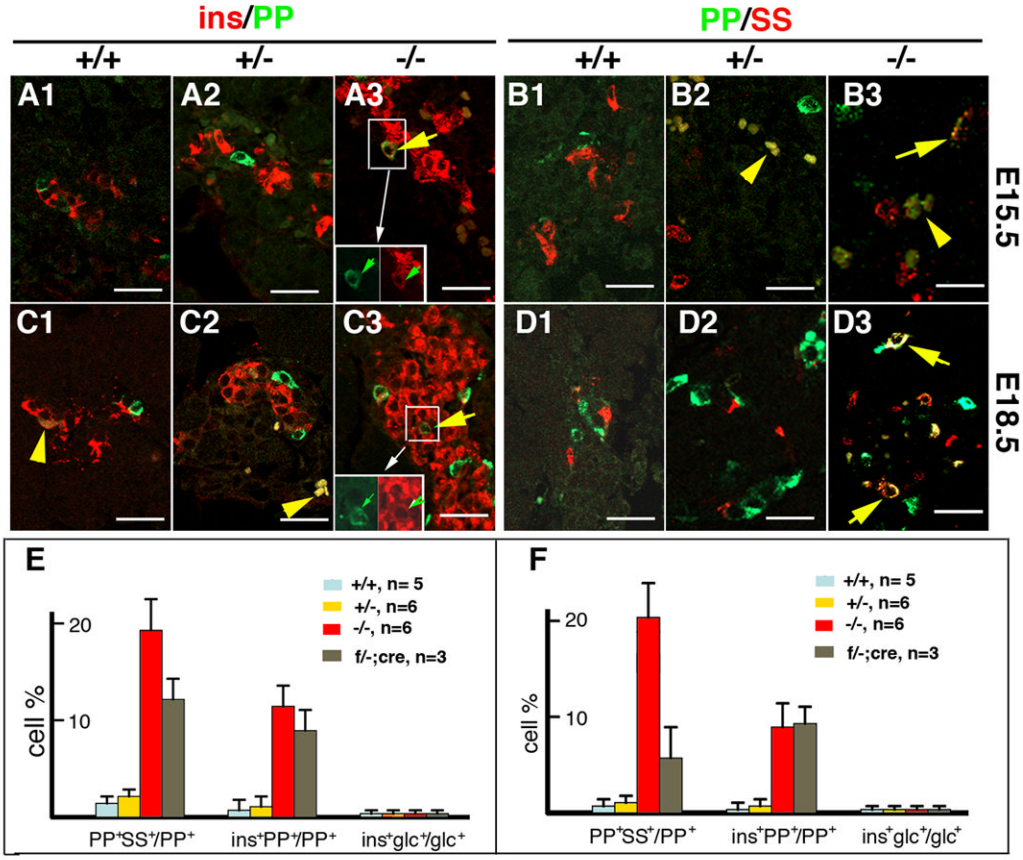

Figure 5. Abnormal hormone-expressing cells are present in *Myt1*$^{-/-}$ embryonic pancreas

The immunofluorecence color of each hormone was labeled on top of the picture). Results from two embryonic stages are shown. (A and B), E15.5. (C and D), E18.5. (A and C), PP and insulin co-staining. (B and D), PP and SS co-staining. Tissue genotypes were marked on top of each column. The double positive cells (yellow arrows) show a yellow color in the cytoplasm, resulted from overlapping red and green. Blood cells also give yellow color (yellow arrowheads), yet they can be distinguished from the double positive cells by their specific appearance. Insets in A3 and C3 showed boxed regions within each panel, split as green and red channel (two small green arrows point to a same cell). (E and F), quantification results of abnormal cell types. Each cell type is represented as the percent of total PP or glucagon-expressing cells. Tissues of different genotypes are represented as different colored bars. f/−;Cre refers to *Myt1*$^{fl/-}$*;Pdx1Cre*. Ins: insulin. Glc: glucagon. Scale bars=20 μm.

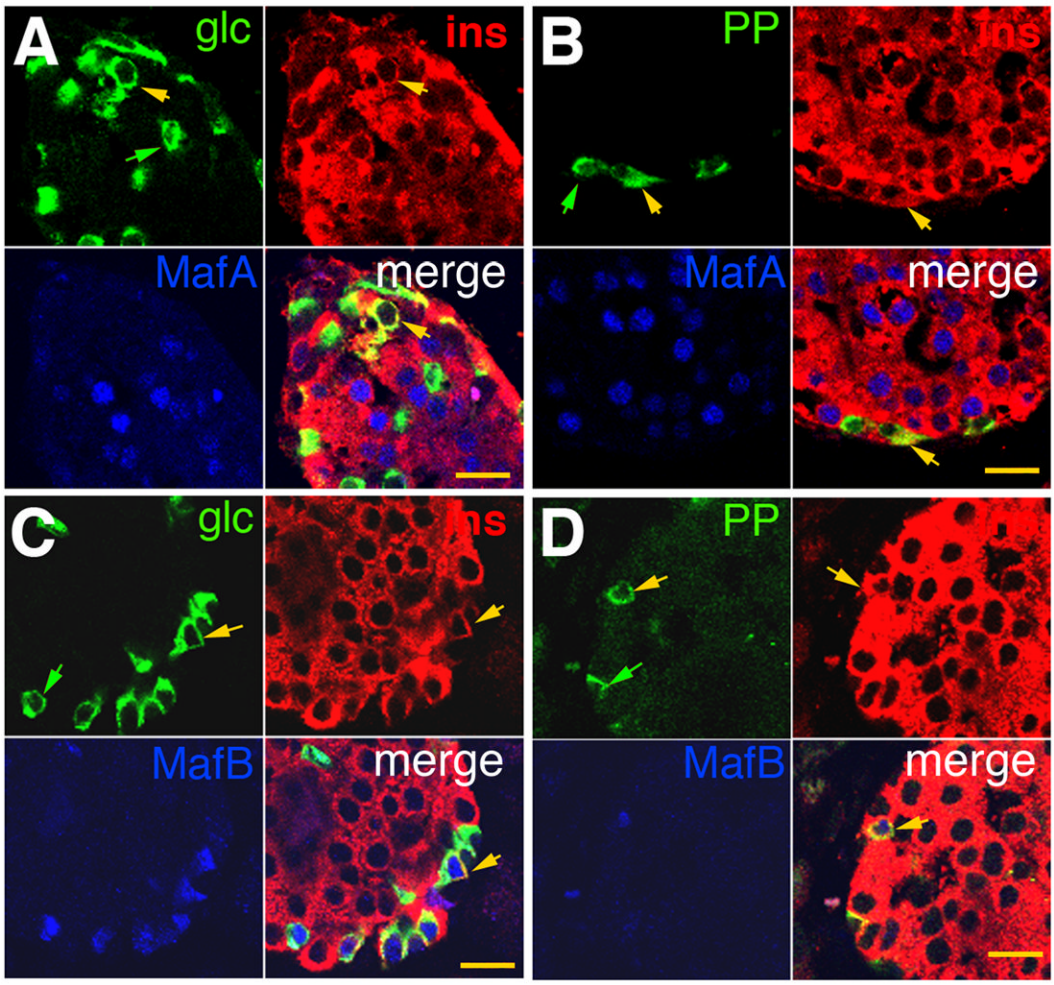

Figure 6. Insulin^{+}/PP^{+} and glucagon^{+}/insulin^{+} cells in the adult *Myt1*$^{fl/-}$;*Pdx1-Cre* islets are not mature β cells

Three channels representing two hormones and a transcription factor staining and overlay (merge) are shown in panels A, B, C, and D. The overlay pinpoints the position of hormone co-expressing cells. In all panels, red fluorescence is a result of insulin staining. Green or blue fluorescence represents staining against different hormone(s) or transcription factor(s), as labeled within each panel. Green arrows, single hormone^{+} cells (internal controls). Yellow arrows, double hormone positive cells. Scale bars=10 μm.

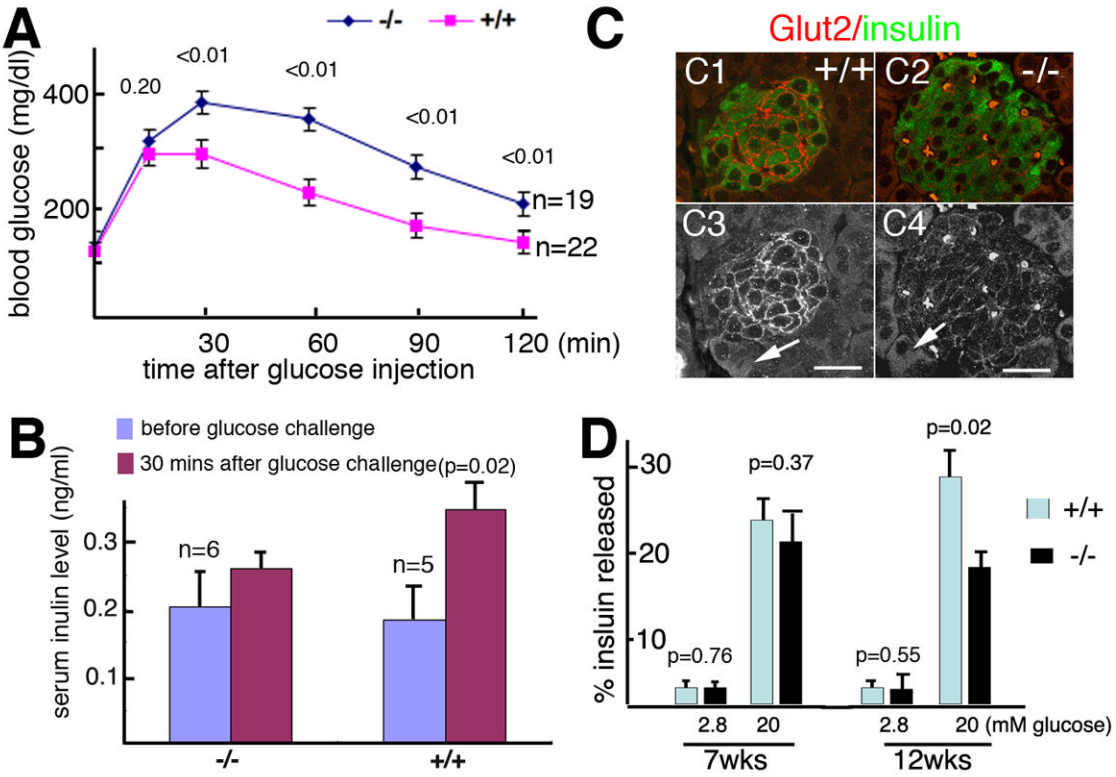

Figure 7. Pancreas-specific *Myt1* mutant males have impaired glucose tolerance, insulin secretion defects, and reduced Glut2 expression in the islets

(A), GTT test results in pancreas-specific *Myt1* mutants at 8 weeks of age (a p-value was marked above each time point of assay). (B), glucose-induced insulin release in wild type and pancreas-specific $Myt1^{-/-}$ mutant animals (10 weeks of age). The serum insulin levels are shown. The p-value is calculated according to the percent of serum insulin increase of each animal, before and after glucose challenge. (C), Glut2 expression in wild type and mutant adult islets (3 month old). Insulin staining located the islet position (C3 and C4 correspond to the red Glut2 channel in C1 and C2, respectively). The two white arrows in C3 and C4 point to two acinar cells that do not express Glut2. Also note the presence of blood cells in panel C2 (appeared red) and C4 (appeared white). (D), GSIS in isolated islets from $Myt1^{fox/-}$;*Pdx1-Cre* animals at two ages. Islets from wild type animals were used as controls. The p-values are marked above each data set. Scale bars=20 μm.

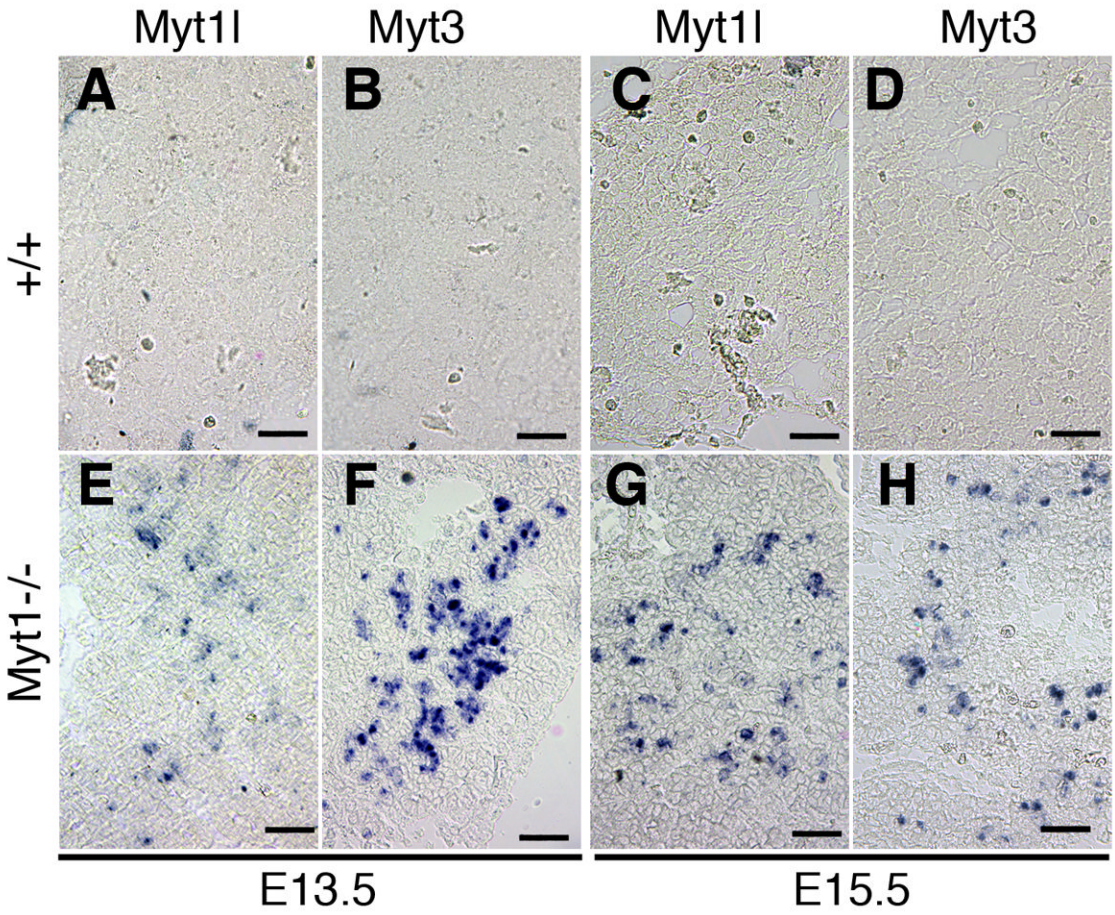

Figure 8. *Myt1l* and *Myt3* are expressed in the *Myt1* mutant pancreas

In situ hybridization results are shown. (A-D), wild type pancreas. (E-H), *Myt1*$^{-/-}$ pancreas. (A, B, E and F), E13.5 pancreatic sections. (C, D, G, and H), E15.5 pancreatic sections. (A, E, C, and G), *Myt1L* cRNA probes. (B, F, D, and H), *Myt3* cRNA probes. Scale bars=20 μm.

Table 1

The % of hormone-expressing cells that express Myt1 (n=4, mean ± standard deviation).

stage	Myt1$^+$ ins$^+$/ins$^+$	Myt1$^+$ glc$^+$/glc$^+$	Myt1$^+$ PP$^+$/PP$^+$	Myt1$^+$ SS$^+$/SS$^+$
E10.5	100±0	100±0	NA	NA
E13.5	91.4±7.6	83.2±4.8	NA	NA
E15.5	93.2±4.1	95.2±3.6	97.8±5.1	94.1 ±3.8
E18.5	94.3±2.9	96.1±4.2	96.3±3.2	97.4±4.3
12wks	58.6±6.7	93.9±4.6	96.6±5.1	96.3±5.9

Table 2

% cells that co-express Myt1 and Ngn3 over all Myt1 and/or Ngn3+ cells mean ± standard deviation).

Stages/cell types	$Myt1^{-}Ngn3^{+}$	$Myt1^{+}Ngn3^{+}$	$Myt1^{+}Ngn3^{-}$
E10.5 (n=12)	8.4±2.9	16.6±3.1	75.1±4.7
E13.5 (n=7)	19.8±3.1	61.5±4.8	18.1±5.6
E15.5 (n=7)	15.3±2.7	31.2±4.2	54.0±3.9