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Transcription Factor Glis3, a Novel Critical Player in the Regulation of Pancreatic β-Cell Development and Insulin Gene Expression▽

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In this study, we report that the Krüppel-like zinc finger transcription factor Gli-similar 3 (Glis3) is induced during the secondary transition of pancreatic development, a stage of cell lineage specification and extensive patterning, and that *Glis3*$^{zf/zf}$ mutant mice develop neonatal diabetes, evidenced by hyperglycemia and hypoinsulinemia. The *Glis3*$^{zf/zf}$ mutant mouse pancreas shows a dramatic loss of β and δ cells, contrasting a smaller relative loss of α, PP, and ε cells. In addition, *Glis3*$^{zf/zf}$ mutant mice develop ductal cysts, while no significant changes were observed in acini. Gene expression profiling and immunofluorescent staining demonstrated that the expression of pancreatic hormones and several transcription factors important in endocrine cell development, including Ngn3, MafA, and Pdx1, were significantly decreased in the developing pancreata of *Glis3*$^{zf/zf}$ mutant mice. The population of pancreatic progenitors appears not to be greatly affected in *Glis3*$^{zf/zf}$ mutant mice; however, the number of neurogenin 3 (Ngn3)-positive endocrine cell progenitors is significantly reduced. Our study indicates that Glis3 plays a key role in cell lineage specification, particularly in the development of mature pancreatic β cells. In addition, we provide evidence that Glis3 regulates insulin gene expression through two Glis-binding sites in its proximal promoter, indicating that Glis3 also regulates β-cell function.

Proteins Glis1 to -3 constitute a subfamily of Krüppel-like zinc finger transcriptional regulators that share a highly conserved five-C_2H_2-type zinc finger domain with members of the Gli and Zic subfamilies (6, 25, 27, 30–32, 35, 38, 39, 43, 56). Glis1 to -3 regulate gene transcription by binding specific DNA sequences referred to as Glis-binding sites (Glis-BS) in promoter regulatory regions of target genes (10, 30, 31). Although their precise physiological functions are still poorly understood, genetic studies have implicated Glis1 to -3 in several pathologies (7, 8, 24, 29, 33, 39, 45).

Glis3 is abundantly expressed in the adult kidney, pituitary, pancreas, uterus, and thyroid gland (31, 45). During mouse embryonic development, Glis3 is expressed in a spatiotemporal pattern suggesting that Glis3 regulates gene expression at specific stages during development (31). Genetic alterations in the human *GLIS3* gene have been linked to a rare syndrome characterized by neonatal diabetes and congenital hypothyroidism (NDH) (45, 50). Depending on the nature of the *GLIS3* mutation, NDH patients can also display facial abnormalities, glaucoma, liver fibrosis, and polycystic kidney disease. Recently, a genome-wide association study identified the *GLIS3* gene as a susceptibility locus for type 1 diabetes (8). These studies, together with evidence that Glis3 is expressed in pancreatic β cells, suggest that Glis3 has an important regulatory role in the pancreas.

Although major advances have been made in understanding pancreatic development, many of the molecular mechanisms that regulate progenitor cell dynamics and cell differentiation are still not precisely understood (1, 18, 19, 21, 26, 28, 37). At approximately embryonic day 9 (E9) of mouse embryogenesis, the pancreas first appears from distinct ventral and dorsal anlagen as evaginations of the distal foregut endoderm (21, 36). The buds grow and initiate branching morphogenesis at about E11.5. Early multipotent pancreatic progenitors, marked by Pdx1, Ptf1a, Nkx2.2, and Cpa1 expression (12, 14, 41, 57), are the source of all differentiated cells of the exocrine, ductal, and endocrine cell lineages. Lineage determination is a complex process that involves many transcription factors and signaling pathways. Induction of Ngn3 marks the differentiation of pancreatic progenitors into proendocrine progenitors (15, 17, 22, 36, 51). Differentiation into the different endocrine cell lineages involves the induction of a combination of additional transcription factors, including Myt1, NeuroD, Isl1, Pax4, Pax6, and Arx (2, 13, 22, 34, 36, 37, 48, 49). Defects in the expression or activity of these transcription factors in mice and humans often result in abnormal pancreatic development and function that can lead to diabetes.

To obtain greater insights into the physiological and molecular functions of Glis3, we recently generated *Glis3*$^{zf/zf}$ mutant mice that are deficient in Glis3 transactivating activity (24). In this study, we characterize the pancreatic phenotype of these mice and analyze the role of Glis3 in pancreatic development. We demonstrate that, in addition to cyst formation in the

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pancreatic ducts, *Glis3*$^{zf/zf}$ mutant mice develop neonatal diabetes that is associated with an almost total loss of β cells. We provide evidence which indicates that Glis3 plays a key role in cell lineage specification, particularly in the development of mature pancreatic β cells. We further identify Glis3 as a regulator of insulin 2 gene expression. Our study shows that Glis3 has multiple functions in the pancreas and suggests that Glis3 might provide a new therapeutic target to intervene in diabetes.

MATERIALS AND METHODS

***Glis3*$^{zf/zf}$ mutant mice.** *Glis3*$^{zf/zf}$ mutant mice, in which a 3.5-kb region that includes exon 4 and parts of introns 3 and 4 was deleted, were described previously (24). This deletion results in the removal of the fifth zinc finger motif (ZF5) of Glis3, disruption of its DNA-binding capacity, and impairment of Glis3 transcriptional activation function (9, 24). All of our animal studies followed the guidelines outlined in the NIH Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Institutional Animal Care and Use Committee at the National Institute of Environmental Health Sciences (NIEHS).

Histopathological evaluation. Histopathological evaluation of male and female wild-type (WT) and Glis3$^{zf/+}$ and *Glis3*$^{zf/zf}$ mutant mice was performed on postnatal day 1 (PND1) and PND3. Tissues were collected and fixed by immersion in 10% neutral buffered formalin or 4% paraformaldehyde–phosphate-buffered saline for 24 h, subsequently embedded in paraffin, and sectioned at 5 μm. The sections were stained with hematoxylin and eosin for histopathological evaluation.

Immunohistochemical analysis. Formalin-fixed/paraffin-embedded or cryogenic sections were used for immunohistochemistry or immunofluorescence staining. For cryosections, embryos were fixed overnight at 4°C in 4% paraformaldehyde, transferred to 30% sucrose for 30 h, and subsequently embedded in OCT (Tissue-Tek, Hatfield, PA). Antibodies against insulin, cadherin 1 (E-cadherin), α-acetylated tubulin, and amylase were purchased from Sigma (St. Louis, MO). Fluorescein isothiocyanate-labeled *Dolichos biflorus* agglutinin (DBA) was obtained from Vector Laboratories (Burlingame, CA). Antibodies against glucagon, somatostatin, and pancreatic polypeptide were from Dako (Carpinteria, CA). Rabbit anti-Hnf6 was from Santa Cruz Biotech (Santa Cruz, CA), rabbit anti-osteopontin was from R&D Systems (Minneapolis, MN), rabbit anti-Sox9 and anti-Glut2 were from Chemicon (Billerica, MA), and rabbit anti-MafA was from Bethyl Laboratories (Montgomery, TX). Mouse anti-Nkx6.1 (AB2022) and rabbit anti-Ngn3 (44) antibodies were kindly provided by Christopher Newgard, Duke University, and Michael German, University of California, San Francisco, respectively. Rabbit and guinea pig anti-Pdx1 antibodies were from Abcam (Cambridge, MA), and rabbit anti-Cpa1 was from Serotec (Raleigh, NC). Alexa Fluor-conjugated secondary antibodies were purchased from Molecular Probes (Carlsbad, CA). Fluorescence was observed in a Leica DMRBE microscope (Leica, Wetzlar, Germany).

In situ hybridization. In situ hybridization was performed with cryosections of pancreas and embryos as described previously (31). The Glis3 probe, encoding the region from Q204 to S507, was generated by PCR and subsequently cloned into pGEM Teasy (Promega, Madison, WI). Plasmid DNA from forward and reverse clones was then linearized with SpeI to generate T7-generated sense and antisense transcripts, respectively, and labeled riboprobe produced with digoxigenin-substituted UTP (Roche, Indianapolis, IN). Sense probes did not show any signal (data not shown).

Glucose and insulin assays. Blood and urine were collected from PND3 pups. Glucose levels were measured with Multistix 10 SG from Bayer. Blood insulin levels were measured with an enzyme-linked immunosorbent assay kit from LINCO Research (St. Charles, MO) according to the manufacturer's instructions.

Microarray analysis. Microarray analyses were carried out by the NIEHS Microarray Group with Agilent whole-genome mouse oligonucleotide arrays (Agilent Technologies, Palo Alto, CA) as described previously (23). Total RNA was isolated from the pancreata of PND3 and E15.5 WT and *Glis3*$^{zf/zf}$ mutant mice with a Qiagen RNeasy Mini kit and subsequently amplified in accordance with the Agilent Low RNA Input Fluorescent Linear Amplification kit protocol. RNAs from three or four individual mice of each strain were analyzed in duplicate. Hybridizations were performed as described previously. Data were obtained with the Agilent Feature Extraction software (v7.1) by using defaults for all parameters. Images and GEML files, including error and *P* values, were exported from the Agilent Feature Extraction software and deposited into Rosetta Resolver (version 3.2, build 3.2.2.0.33; Rosetta Biosoftware, Kirkland, WA). The resultant ratio profiles were combined into ratio experiments as described previously (23). Intensity plots were generated for each ratio experiment, and genes were considered "signature genes" if the *P* value was less than 0.001.

Real-time QRT-PCR analysis. Quantitative reverse transcription-PCRs (QRT-PCRs) were carried out in triplicate in a 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) as previously described (23). All results were normalized to an internal control, either the 18S or the glyceraldehyde-3-phosphate dehydrogenase transcript. Primers were designed with Primer Express 2.0 software and synthesized at Sigma/Genosys (St. Louis, MO). The primers and probes used in this study are shown in Table 1.

Generation of reporter and expression constructs. To generate mIns2 and hINS reporter constructs, the indicated regions of either the mouse insulin 2 or human insulin promoter were amplified by PCR from genomic DNA (Promega) and ligated into the pGL4.10 luciferase reporter vector (Promega) to generate mIns2(−696)-Luc or hINS(−700)-Luc. Site-directed mutagenesis of the two putative Glis-binding sites within the mouse *Ins2* promoter was carried out with the QuikChange site-directed mutagenesis kit (Stratagene) in accordance with the manufacturer's protocol. In the following sequences, the mutated bases are underlined: Glis-BS(−263), 5′-GGAACAATGTCTTCTGCTGTGAAC; Glis-BS(−99), 5′-CTGC-TGACCTACTTCACCTGGAGCCC. The Glis3 expression vector p3xFlag-CMV-Glis3 was described previously (9). Plasmids p3xFlag-CMV-Glis3ΔN302 and p3xFlag-CMV-Glis3ΔC748 were generated by PCR amplification of the respective fragments and insertion into the p3xFlag-CMV10 expression vector (Sigma). All constructs were verified by restriction enzyme analysis and DNA sequencing.

Electrophoretic mobility shift assay (EMSA). Binding of Glis3 to ^{32}P-labeled Glis-BS oligonucleotides was performed as described previously (9). In addition to the Glis-BS consensus sequence, binding of Glis3 to the putative Glis-BS in the mouse *Ins2* proximal promoter region at −84 to −109 (Glis-BS1, 5′-CTGC TGACCTACCCCACCTGGAGCCC), −253 to −276 (Glis-BS2, 5′-GGAACA ATGTCCCCTGCTGTGAAC), and their mutant oligonucleotides (shown above) was examined.

Cells. Rat insulinoma INS-1 (832/13) cells were a generous gift from H. Hohmeier (Duke University) and were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol.

Reporter assays. Cells were plated in 12-well dishes at 1×10^5/well, incubated for 24 h at 37°C, and subsequently cotransfected with 1 μg of the indicated reporter, 0.3 μg pCMV-β-galactosidase, and 0.5 μg of the indicated expression vector in Opti-MEM (Gibco) with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Each transfection cocktail was used to transfect triplicate sets of wells. Cells were harvested 24 h later, and reporter activity was measured with a luciferase assay kit (Promega). β-Galactosidase levels were measured with a luminometric β-galactosidase detection kit (Clontech) by following the manufacturer's protocol. Each data point was assayed in triplicate, and each experiment was performed at least twice. Luciferase data were normalized to β-galactosidase activity and are presented as the mean ± the standard error of the mean. Statistical significance was determined by analysis of variance and Tukey-Kramer comparison tests with InStat software (GraphPad Software Inc.).

Microarray data accession number. The microarray data discussed in this study have been deposited in the NCBI Gene Expression Omnibus as GSE18172 (GEO, http://www.ncbi.nlm.nih.gov/geo/).

RESULTS

Glis3 is expressed during pancreatic development. Recent human genetic studies implicated *GLIS3* in a syndrome with NDH and suggested a regulatory function for *GLIS3* in the pancreas (8, 45, 50). To gain greater insights into the role of Glis3 in the pancreas and neonatal diabetes, we analyzed Glis3 expression during early mouse pancreatic development. QRT-PCR analysis of Glis3 expression during E11.5 to E18.5 of pancreatic development showed that Glis3 expression was relatively low at E11.5 but significantly increased at E12.5, which is at the beginning of the second transition of pancreatic development, and remained high through E18.5 (Fig. 1A). In situ

TABLE 1. QRT-PCR primers and probes used in this study

Gene	5′→3′ sequence		
	Forward primer	Reverse primer	Probe[a]
Ins1	ACCATCAGCAAGCAGGTCAT	CACTTGTGGGTCCTCCACTT	
Ins2	CAGCAAGCAGGAAGCCTATC	TTGTGCCACTTGTGGGTCCT	
Gcg	AGGCCGAGGAAGGCGA	TGCCTGCGGCCGAGT	TCCCAGAAGAAGTCGCCATTGCTGA
Sst	CCACCGGGAAACAGGAACTG	GGGCCAGGAGTTAAGGAAGA	
Ppy	TAGCTCAGCACACAGGATGG	GCCTGGTCAGTGTGTTGATG	
Ghrl	GGCAGGCTCCAGCTTCCT	GGCTTCTTGGATTCCTTTCTCTG	AGCCCAGAGCACCAGAAAGCCCA
Isl1	ACATGGGCGATCCACCAA	TCGTGAATTTGATTGCCGC	ACCAACACACAGGGAAATCAGACGTTTTTTT
Ngn3	TTCTTTTGAGTCGGGAGAACTAGG	GGGACACTTGGATGGTGAGC	TGGCGCCTCATCCCTTGGATG
Pax4	CGGGACAAGCCGAGGC	CGGCCACTGAATCTGGATA	TGGAGAAAGAGTTTCAGCGTGGGCA
Pax6	CCACCACACCTGTCTCCTCCT	TTGGTGAGGGCGGTGTCT	CATCAGGTTCCATGTTGGGCCGAA
Nkx2.2	TCGCTGACCAACACAAAGAC	GCTTTGGAGAAGAGCACTCG	
Nkx6.1	CCCGGAGTGATGCAGAGT	AGAGTTCGGGTCCAGAGGTT	
MafA	CAGCAAGGAGGAGGTCATCC	GCGTAGCCGCGGTTCTT	CTGAAACAGAAGCGGCGCACGC
NeuroD1	TCCGGTGCCGCTGC	GCGAATGGCTATCGAAAGACA	TCGCTGCGAGATCCCCATAGACAACAT
Pdx1	AAATCCACCAAAGCTCACGC	CTCGGGTTCCGCTGTGTAAG	CTCCTGCCCACTGGCCTTTCCA
Abcc8	TTGCTGAAACCGTGGAAGG	GGAGCTTCTGCTGGAACCG	CCATCCGTGCCTTCAGGTACGAGG
Amylase	GCTTATCAGGTCAGAAATTTCG	CATTCCACTTGCGGATAACTG	
Ptf1a	ATCGAGGCACCCGTTCAC	CGATGTGAGCTGTCTCAGGA	
Rat *Glis3*	GTGAAGGCACATTCTTCCAAAGA	GGAGATCTGGATGGAGCTCAGT	CAACAAGCAAGGAAAAAGCTACGGTCCA
Rat *Ins1*	CCTGCTCGTCCTCTGGGAGCCCAAG	CTCCAGTGCCAAGGTCTGAAGATCC	
Rat *Ins2*	CCTGCTCATCCTCTGGGAGCCCCGC	CTCCAGTGCCAAGGTCTGAAGGTCA	

[a] Each probe includes 6-carboxyfluorescein at the 5′ end and 6-carboxytetramethylrhodamine at the 3′ end.

hybridization analysis detected expression of Glis3 mRNA in E11.5 pancreata, as well as at later stages (E12.5, E13.5, E16.5, and E18.5) of pancreatic development (Fig. 1C to G). At E18.5, which is at the beginning of the third transition of pancreatic development, Glis3 expression was restricted to islets and ducts (Fig. 1G). These results suggest that Glis3 may have a critical role early during the second transition of pancreatic development and that its function may be restricted to pancreatic islets and ducts in the maturing pancreas. This conclusion is in agreement with data showing that Glis3 mRNA was highly expressed in βTC (β-cell-like) and PANC1 (pancreatic duct-like) cells, but at low levels in αTC (α-cell-like) cells (Fig. 1B) and is consistent with a previous study showing high expression of Glis3 in β cells (45).

***Glis3*$^{zf/zf}$ mutant mice display overt postnatal diabetes.** To study the physiological functions of Glis3 in the pancreas, we recently generated Glis3 mutant (*Glis3*$^{zf/zf}$) mice in which ZF5 of Glis3 is deleted, yielding a Glis3 defective in its transcriptional activity (9, 24). *Glis3*$^{zf/zf}$ mutant mice have a normal general appearance but die within several days after birth (24). Biochemical analysis showed that blood and urine glucose levels were significantly elevated in *Glis3*$^{zf/zf}$ mutant mice compared to those in WT and heterozygous mice (Fig. 2A and B), while urine pH and ketone levels remained unchanged and the mice did not develop signs of proteinuria (data not shown). Normally, elevation of blood glucose levels induces an increase in circulating insulin; however, serum insulin levels in *Glis3*$^{zf/zf}$ mutant mice were significantly lower than those in WT and *Glis3*$^{+/zf}$ mutant mice (Fig. 2C). The development of hyperglycemia and hypoinsulinemia is consistent with the development of neonatal diabetes in *Glis3*$^{zf/zf}$ mutant mice. This phenotype, together with the greatly shortened life span and the development of polycystic kidneys displayed by *Glis3*$^{zf/zf}$ mutant mice (24), shows a great resemblance to the abnormalities observed in human NDH patients with mutations in the *GLIS3* gene (45, 50). These similarities suggest that the *Glis3*$^{zf/zf}$ mutant mice can serve as a suitable model to study this syndrome.

***Glis3*$^{zf/zf}$ mutant mice display a reduced endocrine compartment.** At PND3, *Glis3*$^{zf/zf}$ mutant mice had developed both ventral and dorsal pancreatic derivatives, acini appeared to have formed normally, and little difference in the size of the pancreas was observed between WT and *Glis3*$^{zf/zf}$ mutant mice (data not shown). The development of hyperglycemia and hypoinsulinemia in *Glis3*$^{zf/zf}$ mutant mice suggested that loss of Glis3 function might result in abnormal β-cell development/function. Although islet structures were present, they were greatly reduced in size (Fig. 2H), morphologically distinct, and less tightly organized than those of WT mice (Fig. 2D to G). These observations suggested that *Glis3*$^{zf/zf}$ mutant mice might exhibit a specific defect in endocrine cell development and maturation, alongside a more subtle effect on pancreatic duct morphology. No morphological differences were noticeable between the pancreata from WT and heterozygous mice (data not shown). These results suggest that the *Glis3*$^{zf/zf}$ mutation is recessive, in agreement with *GLIS3* mutations associated with NDH patients (45).

To determine the influence of the *Glis3* mutation on specific endocrine cell types, sections of pancreas from PND3 mice were examined by immunofluorescence with antibodies against several islet hormones (Fig. 3). This analysis showed that the

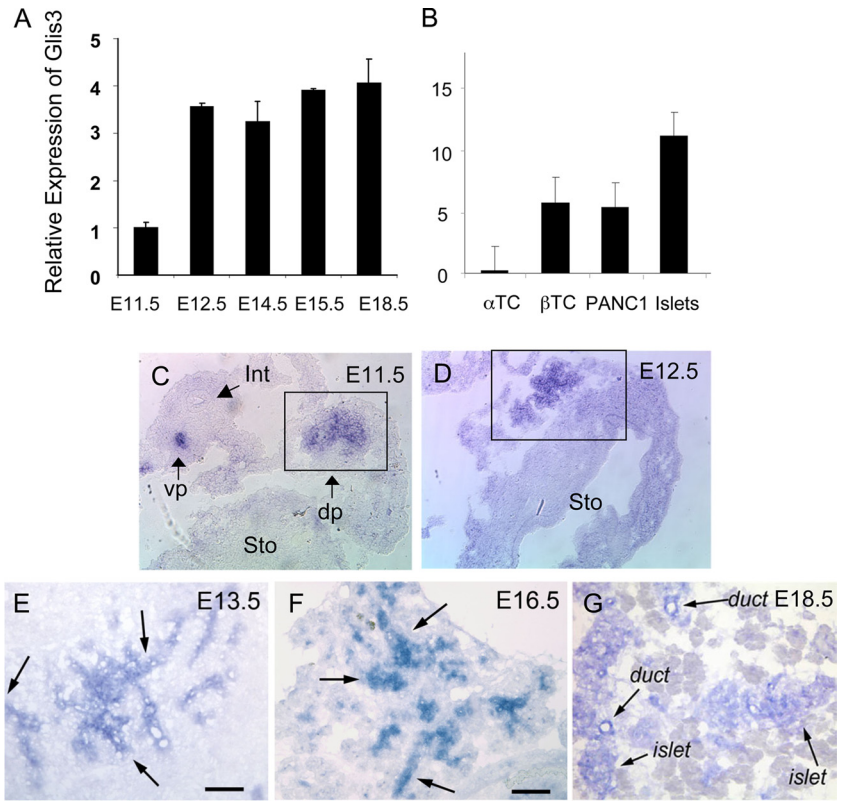

FIG. 1. Expression of Glis3 during pancreatic development. (A) The expression of Glis3 mRNA ($n = 4$) during pancreatic development was examined by QRT-PCR analysis. The expression of Glis3 was normalized to glyceraldehyde-3-phosphate dehydrogenase. (B) Comparison of the relative levels of Glis3 mRNA expression in αTC (pancreatic α-cell-like line), βTC (β-cell-like line), and PANC1 (pancreatic epithelial cell-like line) cell lines and pancreatic islets. The relative expression of Glis3 was analyzed from the Genespeed database (http://genespeed.ccf.org/). (C to G) The expression of Glis3 mRNA in E11.5, E12.5, E13.5, E16.5, and E18.5 pancreata was examined by in situ hybridization. Sto, stomach; vp, ventral pancreas; dp, dorsal pancreas; Int, intestine.

numbers of insulin-positive β cells and somatostatin-positive δ cells were dramatically decreased in pancreatic islets of *Glis3*$^{zf/zf}$ mutant mice compared to those of WT mice, while the numbers of glucagon (Gcg)-positive and pancreatic polypeptide (Ppy)-positive cells were moderately reduced (Fig. 3A to C and G to I). The reduction in insulin-positive β cells was the most striking and probably responsible for the development of hypoinsulinemia and hyperglycemia observed in *Glis3*$^{zf/zf}$ mutant mice. Immunofluorescence staining indicated that the level of hormone expression was not significantly different between the pancreata of heterozygous and WT mice (Fig. 3D to F). These data indicate that Glis3 plays a critical role in the regulation of pancreatic endocrine functions and of β and δ cells in particular.

Pancreatic ducts are dilated in PND3 *Glis3*$^{zf/zf}$ mutant mice. Staining of PND3 pancreatic sections with the lectin DBA, a ductal marker, identified an additional phenotypic change in the pancreata of *Glis3*$^{zf/zf}$ mutant mice. As in the WT pancreas, DBA^{+} cells were prominent in the pancreata of PND3 *Glis3*$^{zf/zf}$ mutant mice, indicating that ductal cell differentiation had proceeded (Fig. 4A to D). However, pancreatic ducts in *Glis3*$^{zf/zf}$ mutant mice were regularly dilated or cystic (Fig. 4B and E). The latter matches the development of a polycystic renal phenotype observed in *Glis3*$^{zf/zf}$ mutant mice (24), as well as in some NDH patients (45). Formation of pancreatic and renal cysts often coincides with and has been linked to defects in the primary cilium structure or in primary cilium-associated signaling pathways (16). Analysis of α-acetylated tubulin, a component of cilia, indicated a significant reduction in the percentage of cells with primary cilia in pancreatic cysts compared to normal ducts (Fig. 4C to F) similar to what we observed in renal tubules (24). These observations are consistent with the hypothesis that the development of pancreatic and renal cysts in *Glis3*$^{zf/zf}$ mutant mice involves a common mechanism.

Loss of Glis3 function affects the expression of endocrine cell-related genes. Because Glis3 functions as a transcriptional regulator (9, 10, 24, 31), one might expect that loss of Glis3 would result in alterations in gene expression profiles in tissues where it is expressed. Therefore, we compared the gene expression profiles in the pancreata of WT and *Glis3*$^{zf/zf}$ mutant mice at E15.5 and PND3 by microarray analysis. These analyses showed that the expression of genes associated with endocrine cell differentiation and function, including several pancreatic hormones and transcription factors, was downregulated in the pancreata of *Glis3*$^{zf/zf}$ mutant mice (Table 2). Very few genes were found to be upregulated in the *Glis3*$^{zf/zf}$ pancreas (data not shown). Although the reduction in the expression of Ins1 and Ins2 mRNA was the most dramatic, the expression of Sst, Ppy, Gcg, and Ghrl was also significantly diminished in the

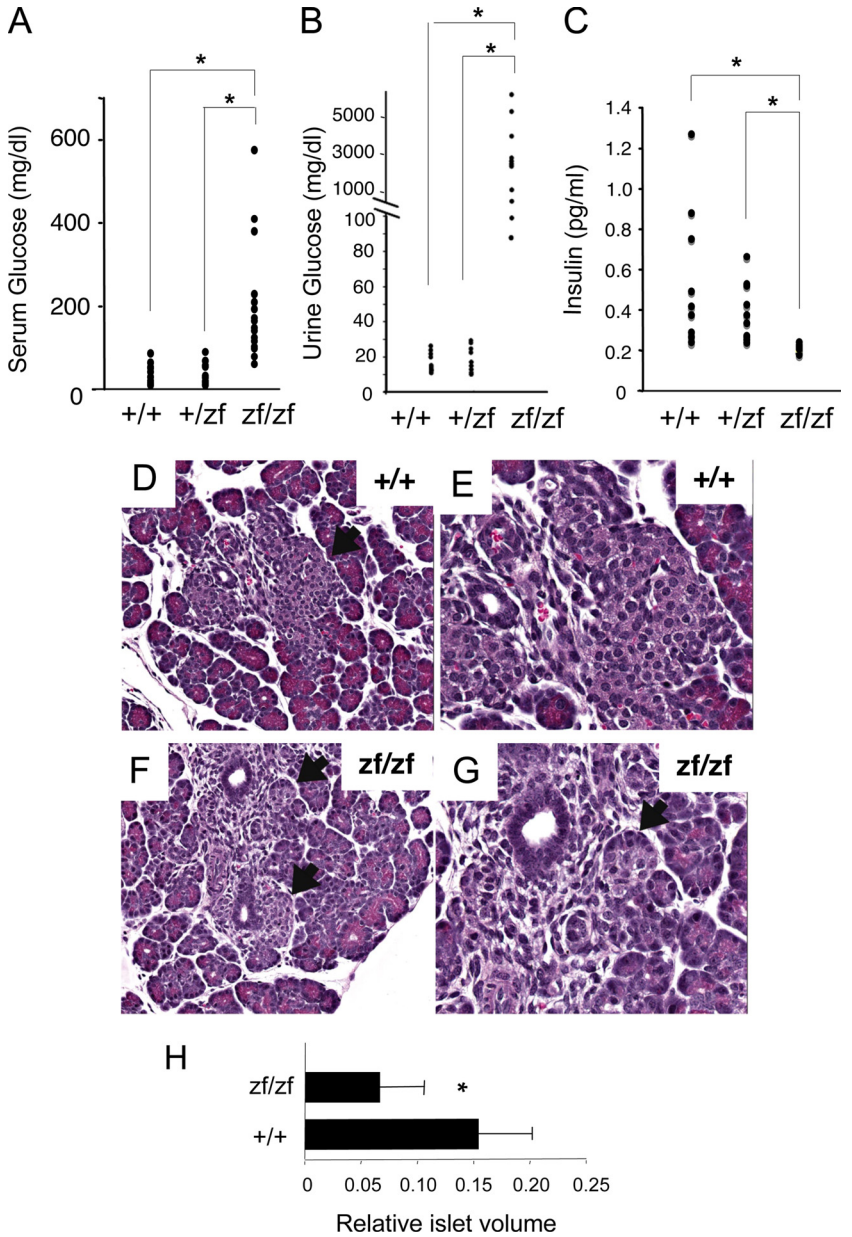

FIG. 2. Development of hyperglycemia and hypoinsulinemia in *Glis3*$^{zf/zf}$ mutant mice. (A) Elevated blood glucose levels in WT (n = 11), *Glis3*$^{+/zf}$ mutant (n = 12), and *Glis3*$^{zf/zf}$ mutant (n = 16) mice. (B) Analysis of urine glucose levels in WT (n = 11), *Glis3*$^{+/zf}$ mutant (n = 9), and *Glis3*$^{zf/zf}$ mutant (n = 11) mice. (C) Comparison of blood insulin levels in WT (n = 10), *Glis3*$^{+/zf}$ mutant (n = 11), and *Glis3*$^{zf/zf}$ mutant (n = 14) mice. (D to G) The size of pancreatic islets is significantly reduced in *Glis3*$^{zf/zf}$ mutant mice. Shown are hematoxylin-and-eosin-stained sections of pancreata from PND3 WT and *Glis3*$^{zf/zf}$ mutant mice. Arrows indicate pancreatic islets. (H) Islet volume was reduced in the PND3 *Glis3*$^{zf/zf}$ mutant pancreas (n = 6) compared to that in the WT pancreas (n = 6). By a systematic random sampling approach, the islet load was assessed in every 10th section throughout the pancreas. An asterisk indicates a P value of <0.02.

E15.5, as well as in the PND3, *Glis3*$^{zf/zf}$ mutant mouse pancreas. The downregulation of the expression of all major pancreatic hormones, including insulin 1 and 2 (*Ins1* and *Ins2*), somatostatin (*Sst*), *Gcg*, *Ppy*, and ghrelin (*Ghrl*), in the PND3 *Glis3*$^{zf/zf}$ mutant pancreas was confirmed by QRT-PCR analysis (Fig. 3J). These observations are consistent with the immunofluorescence analyses shown in Fig. 3. In addition to *Ins1* and *Ins2*, the expression of several other β-cell-associated genes, including islet amyloid polypeptide (*Iapp*), ATP-binding cassette subfamily C8 (*Abcc8*), and *Glut2*, was decreased in *Glis3*$^{zf/zf}$ mutant mice (Table 2). Moreover, several genes reported to be associated with both neuroendocrine and β cells, including those for chromogranin A (*Chga*), synaptotagmin (*Syt*), and secretagogin (*Scgn*), were expressed at reduced levels in the *Glis3*$^{zf/zf}$ mutant pancreas.

In contrast to endocrine markers, the expression of exocrine cell-related genes was generally unchanged in *Glis3*$^{zf/zf}$ mutant mice. For example, no significant alterations were observed in

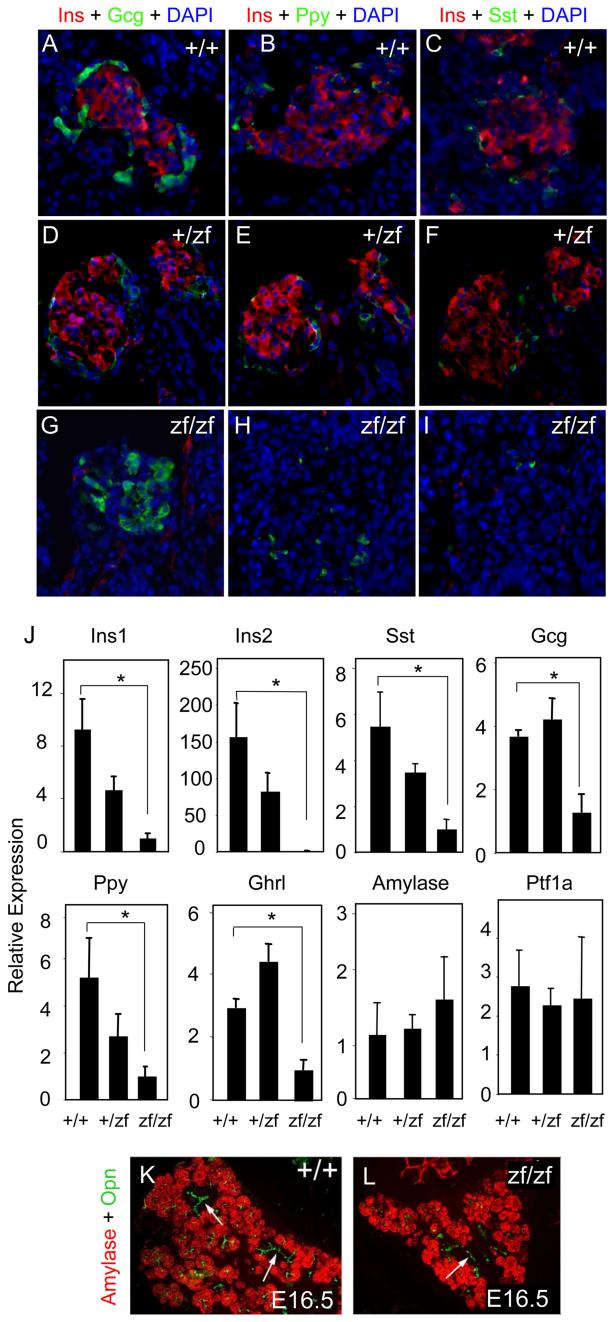

FIG. 3. Loss of Glis3 function causes a dramatic reduction in endocrine cells, particularly β cells. (A to I) Sections of PND3 WT and *Glis3*$^{+/zf}$ and *Glis3*$^{zf/zf}$ mutant pancreata were examined by immunofluorescence with antibodies against insulin (Ins), glucagon (Gcg), pancreatic polypeptide (Ppy), and somatostatin (Sst) as indicated. Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. (J) The expression of several endocrine cell-specific, but not exocrine cell-specific, genes is greatly reduced in *Glis3*$^{zf/zf}$ mutant mice. RNAs isolated from pancreata of PND3 WT and *Glis3*$^{+/zf}$ and *Glis3*$^{zf/zf}$ mutant mice were analyzed by QRT-PCR for the expression of insulin 1 and 2 (Ins1 and Ins2), somatostatin, glucagon, ghrelin (Ghrl), and pancreatic polypeptide mRNAs and the exocrine cell-related genes for amylase and Ptf1a. An asterisk indicates a *P* value of <0.02. (K, L) Expression of amylase is not significantly altered in the *Glis3*$^{zf/zf}$ mutant mouse pancreas. Sections of E16.5 WT and *Glis3*$^{zf/zf}$ mutant mouse pancreata were stained with antibodies against the exocrine enzyme amylase (red) and the ductal marker osteopontin (Opn; green).

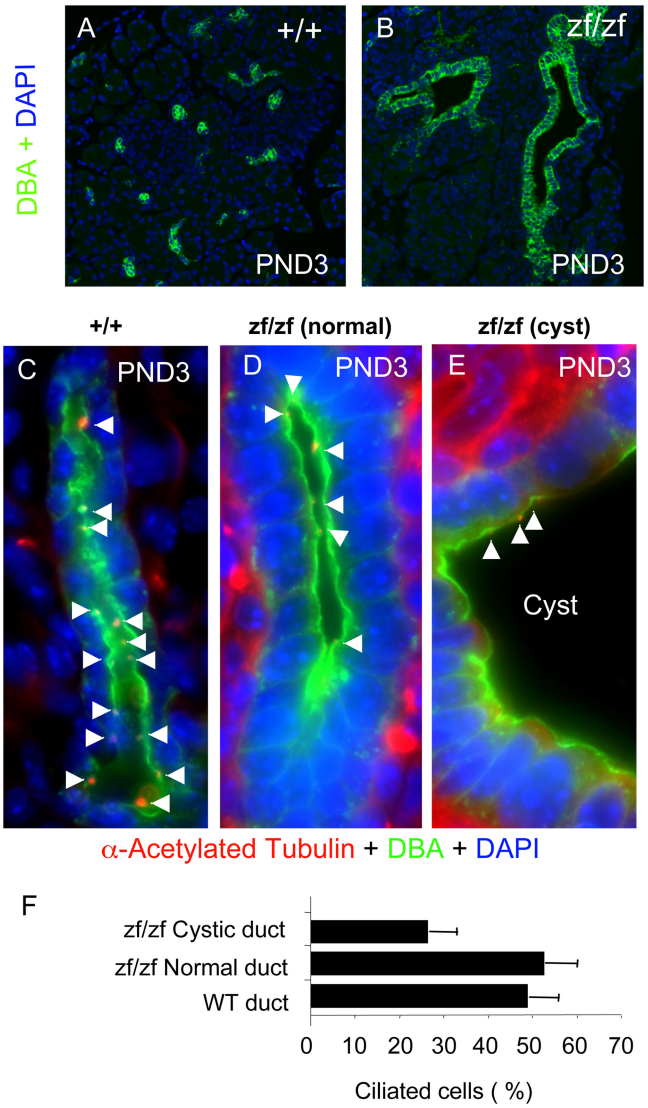

FIG. 4. Development of dilated and cystic pancreatic ducts in *Glis3*$^{zf/zf}$ mutant mice. (A, B) Pancreata from PND3 WT and *Glis3*$^{zf/zf}$ mutant mice were stained with the ductal marker DBA and DAPI. (C to E) Pancreatic ducts and cilia were stained by DBA and anti-α-acetylated tubulin, respectively. Representative images of a pancreatic duct from a PND3 WT mouse (C) and a normal (D) and a cystic (E) pancreatic duct from a PND3 *Glis3*$^{zf/zf}$ mutant mouse are shown. In WT mice, about 50% of the ductal cells were stained positively with anti-α-acetylated tubulin antibody. The number of ciliated cells was greatly reduced in cysts of the *Glis3*$^{zf/zf}$ mutant pancreas. Arrowheads indicate primary cilia. (F) The percentage of ciliated cells is reduced in cystic pancreatic ducts of *Glis3*$^{zf/zf}$ mutant mice. Sections from three different mice in each group were selected randomly and stained with DBA and anti-α-acetylated tubulin antibody. The percentage of DBA-positive cells (n = 50 to 70) containing a primary cilium was calculated.

the expression of the transcription factor Ptf1a, which plays a key role in exocrine cell differentiation, or the exocrine marker amylase (Fig. 3J to L). These findings corroborate the notion that the major effect of the *Glis3* mutation is on the endocrine and not the exocrine compartment of the pancreas.

In addition to the expression of several pancreatic hormones, gene expression profile analysis showed that the ex-

TABLE 2. Genes downregulated in the pancreata of PND3 *Glis3*$^{zf/zf}$ mutant mice[a]

GenBank accession no.	Sequence description	Designation	Fold change at:	
			E15.5	PND3
NM_008386	Insulin I	*Ins1*	−11.8	−17.8
NM_008387	Insulin II	*Ins2*	−38.4	−10.6
NM_021488	Ghrelin	*Ghrl*	−2.2	−5.3
NM_008918	Pancreatic polypeptide	*Ppy*	−3.3	−5.1
NM_021331	Glucose-6-phosphatase, catalytic, 2	*G6pc2*	−4.5	−4.9
NM_009215	Somatostatin	*Sst*	−4.7	−4
AF213386	ATP-binding cassette, subfamily C	*Abcc8*	−3.7	−3.8
NM_030725	Synaptotagmin XIII	*Syt13*	−5	−3.7
X63963	Paired box gene 6	*Pax6*	−3.8	−3.5
NM_007693	Chromogranin A	*Chga*	−10.8	−3.4
NM_013640	Proteasome subunit, beta type 10	*Psmb10*	NC[b]	−3.3
NM_145399	Secretagogin	*Scgn*	−3.2	−3.2
NM_019741	Solute carrier family 2, member 5	*Slc2a5* (*Glut5*)	−1.8	−3.1
NM_011255	Retinol binding protein 4	*Rbp4*	−2.1	−3
NM_010290	Gap junction membrane channel protein alpha 9	*Gja9*	−3.2	−3
NM_011356	Frizzled-related protein	*Frzb*	ND[c]	−3
NM_008240	Forkhead box J1	*Foxj1*	NC	−2.9
NM_013757	Synaptotagmin-like 4	*Sytl4*	−2.1	−2.9
NM_021391	Protein phosphatase 1, r1A	*Ppp1r1a*	−4.1	−2.8
NM_020626	Transmembrane protein 27 (collectrin)	*Tmem27*	−2.9	−2.5
NM_008100	Glucagon	*Gcg*	−2.3	−2.3
NM_009504	Vitamin D receptor	*Vdr*	−2.8	−2.2
AK122226	Regulating synaptic membrane exocytosis 3	*Rims3*	ND	−2.2
NM_023182	Chymotrypsin-like	*Ctrl*	2	−2.2
NM_021459	ISL1 transcription factor	*Isl1*	−1.9	−2.2
NM_009162	Secretogranin V	*Scg5*	−2.2	−2.2
NM_008792	Proprotein convertase subtilisin/kexin type 2	*Pcsk2*	−3.8	−2.1
AK003606	Signal recognition particle 9	*Srp9*	NC	−2.1
AK035727	Slit homolog 3	*Slit3*	−1.4	−2.1
NM_010658	Musculoaponeurotic fibrosarcoma oncogene B	*MafB*	−2.7	−2.1
AK037670	Low-density lipoprotein receptor	*Ldlr*	ND	−2.1
NM_145435	Peptide YY	*Pyy*	−3.1	−2
NM_144955	NK6 transcription factor, locus 1	*Nkx6.1*	−3.5	−1.9
NM_031197	Solute carrier family 2, member 2	*Slc2a2* (*Glut2*)	−2.5	−1.8
NM_010894	Neurogenic differentiation 1	*NeuroD1*	−5.1	−1.6
NM_010491	Islet amyloid polypeptide	*Iapp*	−1.6	−1.4
NM_009719	Neurogenin 3	*Ngn3*	−3.6	ND
NM_011038	Paired box gene 4	*Pax4*	−2.9	NC

[a] Gene expression profiles of pancreata from E15.5 and PND3 WT and *Glis3*$^{zf/zf}$ mutant mice were examined by microarray analysis. Shown is the decrease in gene expression in *Glis3*$^{zf/zf}$ mutant mice (partial list).
[b] NC, no change.
[c] ND, not detectable.

pression of several transcription factors with established roles in the regulation of differentiation of proendocrine progenitors into different endocrine cell lineages, including Ngn3, Nkx6.1, Pax4, Pax6, Isl1, NeuroD1, and MafB, was downregulated in the pancreata of E15.5 and/or PND3 *Glis3*$^{zf/zf}$ mutant mice (Table 2) (1, 14, 18, 19, 26, 28, 37, 57). The reduced expression of several transcription factors was supported by QRT-PCR analysis (Fig. 5A). No significant differences in the expression of these genes were observed between heterozygous and WT mice. Immunofluorescence analysis showed that the number of cells positive for Nkx6.1, Pdx1, and MafA, all of which are β-cell markers, was significantly reduced in pancreatic islets of PND3 *Glis3*$^{zf/zf}$ mutant mice compared to those of WT mice (Fig. 5B to G). Although Pdx1 and Nkx6.1 are expressed in common pancreatic progenitors, they eventually become restricted to mature β cells (40). The mature β-cell marker Glut2 was also dramatically downregulated in the pancreata of *Glis3*$^{zf/zf}$ mutant mice, even within the few remaining Pdx1$^+$ cells (Fig. 5H to M). Staining for the apoptotic marker caspase 3 (data not shown) did not indicate increased apoptosis in endocrine cells from *Glis3*$^{zf/zf}$ mutant mice, suggesting that the reduced expression of these genes is related to impairment in the generation rather than degeneration of β cells. These results are in agreement with the concept that Glis3 plays a critical role in the development of mature β cells.

Endocrine cell progenitors are reduced in *Glis3*$^{zf/zf}$ mutant mice. To obtain further insight into the role of Glis3 in the regulation of β-cell development, we examined the expression pattern of several transcription factors during pancreatic development in *Glis3*$^{zf/zf}$ mutant mice. Considering that Glis3 is induced during E11.5 to E13.5 and is not expressed in mature exocrine cells, we were interested in analyzing the role of Glis3 during this stage of pancreatic development. At E11.5, pancreatic progenitor cells (Pxd1$^+$ Ptf1a$^+$ Cpa1$^+$ Sox9$^+$) rapidly proliferate (36, 57), while at E13.5 they become patterned in a distal/proximal manner, a process that can be monitored by the expression of Pdx1, Sox9, and Cpa1. Comparison of the expression patterns of these proteins in WT and *Glis3*$^{zf/zf}$ mutant

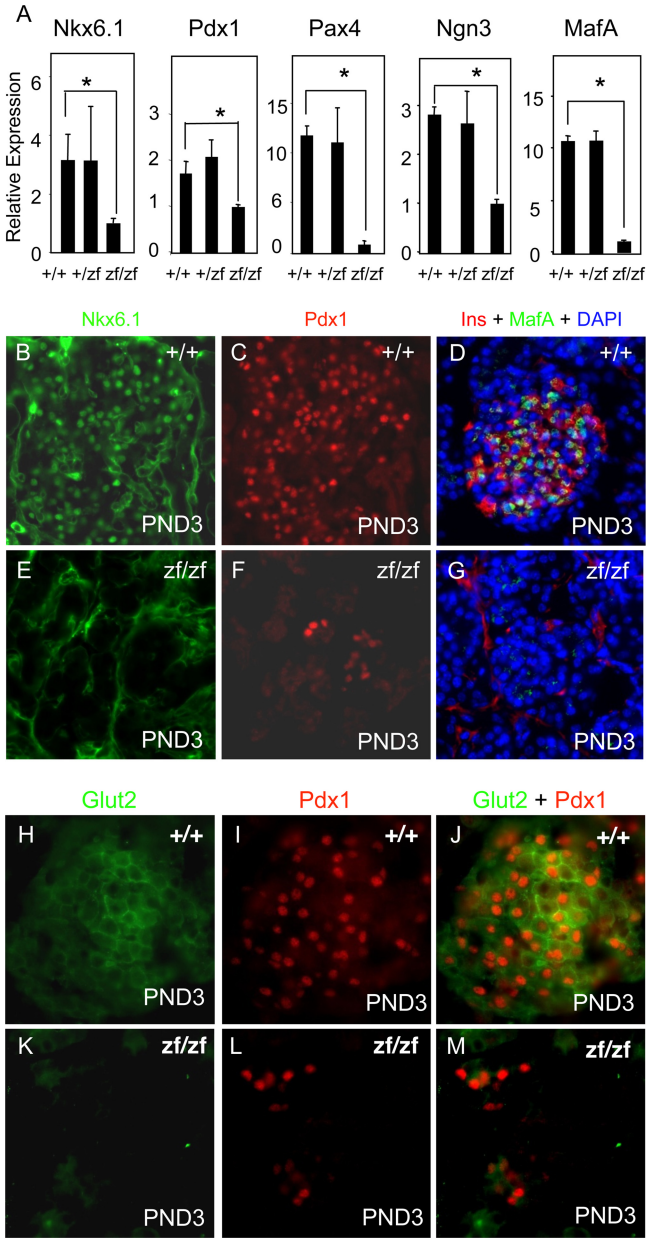

FIG. 5. The expression of several endocrine cell-related transcription factors is significantly reduced in the *Glis3*$^{zf/zf}$ mutant pancreas. (A) The expression of *Nkx6.1*, *Pdx1*, *Pax4*, *Ngn3*, and *MafA*, genes associated with endocrine development, was examined by QRT-PCR in pancreas samples from PND3 WT and *Glis3*$^{+/zf}$ and *Glis3*$^{zf/zf}$ mutant mice ($n = 3$). An asterisk indicates a P value of <0.02. (B to M) Pancreas sections from PND3 WT and *Glis3*$^{zf/zf}$ mutant mice were examined by immunofluorescence with antibodies for Nkx6.1, Pdx1, insulin, MafA, and/or Glut2, as indicated. D, G, J, and M, merged. DAPI, blue staining.

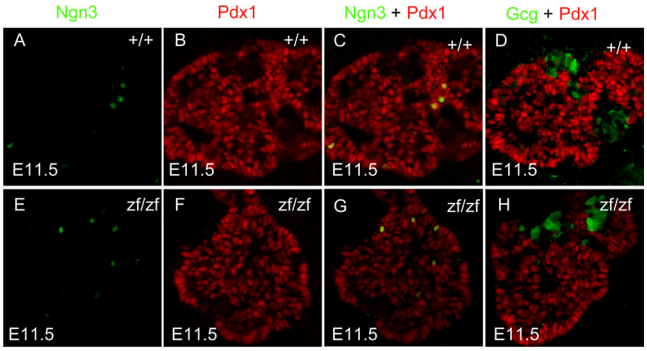

FIG. 6. Analysis of the expression of Pdx1, Ngn3, and glucagon at E11.5 of pancreatic development. (A to H) Expression of Ngn3, Pdx1, and glucagon in pancreas sections from E11.5 *Glis3*$^{zf/zf}$ mutant and WT embryos was examined by immunofluorescent staining. Representative images are shown. No significant differences were observed. Ngn3, green in panels A and E; Pdx1, red in panels B and F; merged in panels C and G. (D, H) Merged glucagon (Gcg; green) and Pdx1 (red) staining of E11.5 pancreas.

pancreata showed no significant difference in the staining pattern for Pdx1 at E11.5 (Fig. 6). Likewise, little difference was observed in the staining patterns for Pdx1 and Sox9 between E13.5 WT and *Glis3*$^{zf/zf}$ mutant pancreata (Fig. 7A to D). In addition, little difference in glucagon staining was observed between the pancreata of WT and *Glis3*$^{zf/zf}$ mutant E13.5 embryos (Fig. 7E and F). At E14.5, Cpa1$^+$ cells formed in a distalized fashion within the branching tips of both WT and *Glis3*$^{zf/zf}$ mutant pancreata (Fig. 8A to F). These observations indicate that Glis3 is not required for pancreatic progenitor maintenance, its patterning, and early endocrine cell development but acts at a later stage of mouse pancreagenesis. We noted, however, that following the secondary transition at E16.5, a stage at which endocrine, exocrine, and ductal cells are organized into islets, acini, and ducts and the expression of Pdx1 becomes confined largely to β cells, the number of Pdx1$^+$ cells was greatly diminished in the *Glis3*$^{zf/zf}$ mutant pancreas (Fig. 7I and J). This decrease correlated with the reduction in Ins$^+$ cells (Fig. 7K and L) and is consistent with the hypothesis that the endocrine (β-cell) compartment is greatly reduced in *Glis3*$^{zf/zf}$ mutant mice.

Because Ngn3 plays a key role in the induction of endocrine cell progenitors and promotes the expression of several more terminal endocrine transcription factors, including NeuroD1, Pax4, Pax6, Nkx6.1, and MafB, we compared the patterns of Ngn3 expression in the pancreata of WT and *Glis3*$^{zf/zf}$ mutant embryos (15, 17, 51, 53). In contrast to E11.5, the number of Ngn3$^+$ cells was greatly reduced in the pancreata of E13.5 *Glis3*$^{zf/zf}$ mutant embryos compared to that in WT embryos (Fig. 7G and H). The difference in the number of Ngn3$^+$ cells was even more dramatic at E16.5 (Fig. 7O and P). Consistent with these data, QRT-PCR analysis showed that the level of Ngn3 mRNA expression was significantly reduced in E13.5 and E15.5 *Glis3*$^{zf/zf}$ mutant pancreata (Fig. 7Q and R). These observations suggest that the *Glis3* mutation may affect the generation of endocrine cell progenitors and implicate Glis3 in the formation of the pool of endocrine cell progenitors.

Regulation of Ngn3 is complex and controlled by multiple transcription factors, including positive regulation by Myt1 and Hnf6 and negative regulation by a Notch/Hes feedback loop (4, 20, 51). At E15.5, no significant difference in Notch expression was observed between the pancreata of WT and *Glis3*$^{zf/zf}$ mutant embryos, while Hnf6 mRNA was slightly elevated (Fig. 7R). Immunohistochemistry showed no significant difference in Hnf6 staining between pancreata from E16.5 WT and *Glis3*$^{zf/zf}$ mutant

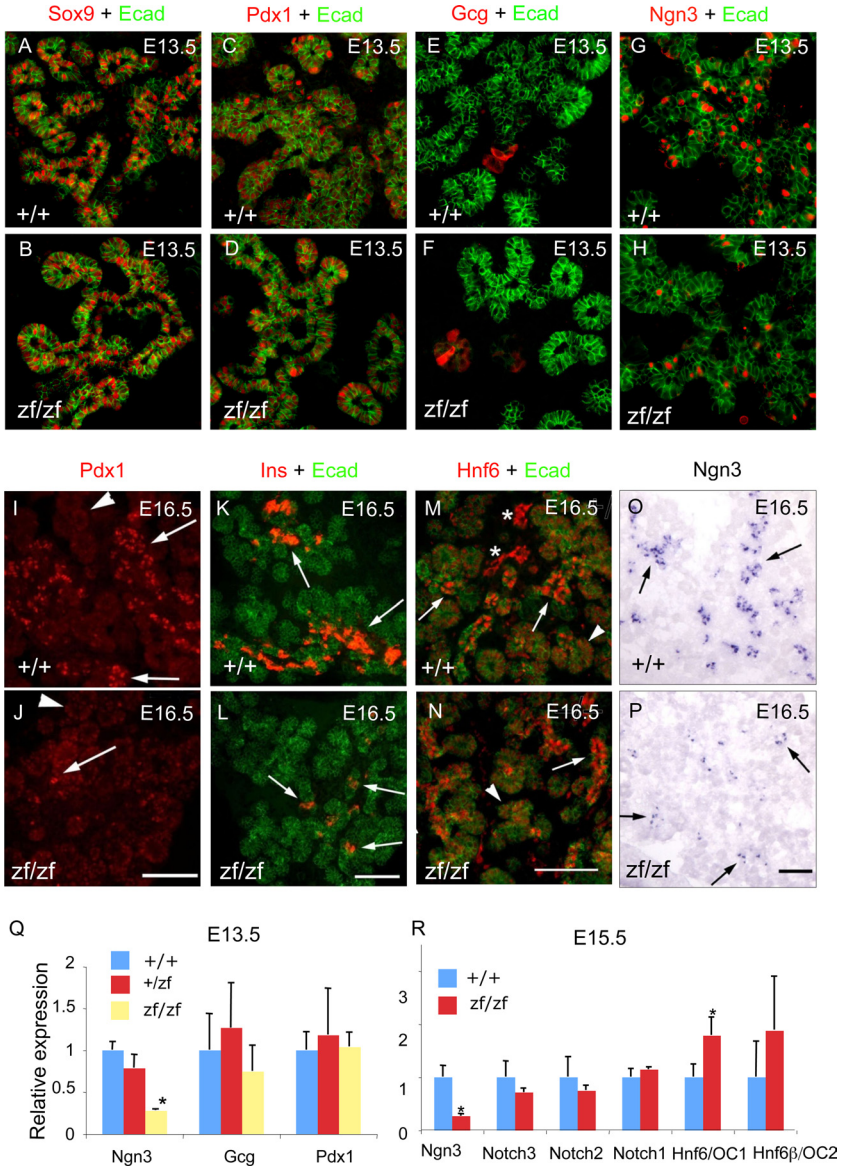

FIG. 7. Comparison of Sox9, Pdx1, Ngn3, and HNF6 expression during the development of WT and *Glis3*$^{zf/zf}$ mutant pancreata. (A to H) The Sox9, Pdx1, glucagon, Ngn3, and E-cadherin proteins were analyzed by immunofluorescence in sections of E13.5 WT and *Glis3*$^{zf/zf}$ mutant pancreata. (I to N) The Pdx1, Ins, and Hnf6 proteins were analyzed in sections of E16.5 WT and *Glis3*$^{zf/zf}$ mutant pancreata by immunofluorescence staining as indicated. (I, J) The numbers of cells expressing high levels of Pdx1 were dramatically decreased in the *Glis3*$^{zf/zf}$ mutant pancreas, as indicated by arrows. Arrowheads indicate cells expressing low levels of Pdx1. (K, L). The number of cells expressing insulin was dramatically decreased in the *Glis3*$^{zf/zf}$ mutant pancreas, as indicated by arrows. (M, N) The expression of Hnf6 is high in the forming ducts (indicated by arrows) and low in the exocrine tissue (indicated by arrowheads); the asterisks indicate nonspecific fluorescence in vessels. (O, P) Ngn3 mRNA expression was examined by in situ hybridization in sections of E16.5 WT and *Glis3*$^{zf/zf}$ mutant pancreata. (Q) The expression of Ngn3, Gcg, and Pdx1 mRNAs was analyzed in samples from WT and *Glis3*$^{+/zf}$ and *Glis3*$^{zf/zf}$ mutant E13.5 embryos ($n = 3$) by QRT-PCR. (R) Expression of Ngn3, Notch1 to -3, Hnf6, and HNF6β mRNAs was compared between E15.5 WT ($n = 3$) and *Glis3*$^{zf/zf}$ mutant ($n = 3$) pancreata. Expression was analyzed by QRT-PCR. An asterisk indicates a P value of <0.05.

embryos (Fig. 7M and N). Moreover, Glis3 expression appeared unaffected in the Hnf6 null pancreas (data not shown).

Regulation of Ins2 expression by Glis3. Our study provides strong evidence of a role for Glis3 in the regulation of β-cell development. Many transcription factors with roles in pancreas development have been reported to exhibit multiple functions. For example, in addition to their role in lineage determination, Pdx1, Ngn3, NeuroD1, and MafA have also been implicated in the regulation of β-cell maturation and maintenance of β-cell function (3, 5, 51). Because loss of Glis3 function affected the expression of *Ins2* the most dramatically, we examined whether Glis3 regulates this gene. Comparison of the human *INS* and mouse *Ins2* proximal promoter region identified two putative Glis-binding sites (Glis-BS1 and Glis-BS2) at −94 and −266 and at −99 and −263, respectively (Fig. 9A and B). EMSA analysis showed that Glis3 was able to bind both of these sites effectively, although not as efficiently as the consensus Glis-BS (Fig. 9C to E). Mutations at these sites abolished Glis3 binding. In addition,

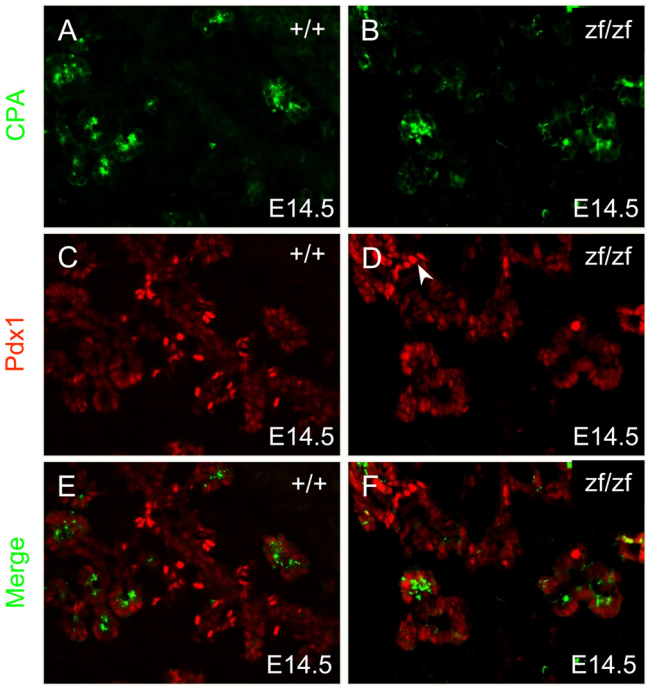

FIG. 8. The expression of Cpa1 was not significantly changed in pancreata of WT and *Glis3*$^{zf/zf}$ mutant E14.5 embryos. Cpa1, green in panels A and B; Pdx1, red in panels C and D; merged in panels E and F.

Glis3 was able to induce *Ins2* and *INS* promoter activity in rat insulinoma INS-1 832/13 cells (Fig. 9F). A C-terminal deletion mutant of Glis3 (Glis3ΔC748) that lacks transactivation function exhibited a greatly diminished ability to activate the *Ins2* and *INS* promoter (Fig. 9F), while the N-truncated mutant Glis3ΔN302 was more effective in activating the *Ins2* promoter (Fig. 9G). The latter is in agreement with previous observations showing increased transactivating activity of N-terminal deletion mutants of Glis3 (31), which may suggest the presence of a repressor domain at the N terminus of Glis3. Point mutations in only one of the two Glis-BS reduced Glis3-mediated activation only partially (Fig. 9G, right panel); however, mutations in both sites almost totally abolished this activity and also reduced basal activation of the *Ins2* promoter by endogenous Glis3 (Fig. 9G, left panel). Again, Glis3ΔN302 was more active. These results strongly support the conclusion that Glis3 functions as a transcriptional regulator of *Ins2* gene expression and suggest that both Glis-BS are important in this regulation. This was further supported by data showing that exogenous expression of Glis3 in INS-1 832/13 cells enhanced the expression of endogenous *Ins2* expression (Fig. 9H). Although another potential Glis-BS was found at −342 to −348, Glis3 did not bind this sequence and mutation of this site did not influence Glis3-mediated activation of the *Ins2* promoter (data not shown), suggesting that this site is not a functional Glis3-binding site.

DISCUSSION

We recently reported that *Glis3*$^{zf/zf}$ mutant mice, which are defective in their transcriptional activation function, have a very short life span and develop polycystic kidney disease (24). In this study, we demonstrate that these mice also display hyperglycemia and hypoinsulinemia, consistent with the development of neonatal diabetes. During revision of the manuscript, a similar phenotype was reported in an alternate Glis3 mutant mouse model (52). These phenotypic changes resemble the abnormalities observed in NDH patients with mutations in *GLIS3* (45, 50) and suggest that *GLIS3* plays a critical role in the regulation of pancreatic endocrine functions and the development of type 1 diabetes. The latter is supported by a recent report identifying the *GLIS3* gene as a susceptibility locus for type 1 diabetes (8). In this study, we further demonstrate that Glis3 plays a critical role in cell lineage specification, particularly the development of β cells, and in the regulation of mature β-cell function. In addition, we show that Glis3 is important in maintaining normal functions in ductal epithelial cells but does not appear to have a major role in the development or function of acini.

Analysis of Glis3 expression during pancreatic development showed that Glis3 mRNA was detectable at E11.5 and expressed at increased levels at E12.5 and later stages of pancreatic development. At E18.5, Glis3 is particularly highly expressed in ductal cells and islets but was not detectable in acini, suggesting that Glis3 functions as a transcriptional regulator particularly in endocrine cell progenitors, β cells, and ductal cells. Immunohistological analysis of the pancreata of PND3 *Glis3*$^{zf/zf}$ mutant mice showed that although the size of the pancreas is not significantly different, the islets are considerably smaller than those of WT mice. Pancreatic acini develop normally and exocrine markers are normally expressed in *Glis3*$^{zf/zf}$ mutant mice. The reduced islet size was largely due to a dramatic decline in the number of fully differentiated β cells, as indicated by the reduction in insulin-, Pdx1-, MafA-, and Glut2-positive cells, as well in the expression of several other β-cell selective genes. This decrease in β cells explains the development of diabetes observed in *Glis3*$^{zf/zf}$ mutant mice. These observations suggest that *Glis3*$^{zf/zf}$ mutant mice exhibit a specific defect in endocrine, rather than exocrine, cell development and indicate that Glis3 is a key factor in regulating the generation and/or maintenance of β cells. Analysis of pancreatic sections for active caspase 3 did not indicate increased apoptosis in the pancreata of E13.5 or E15.5 *Glis3*$^{zf/zf}$ mutant mouse embryos or in the islets of PND3 *Glis3*$^{zf/zf}$ mutant mice. This, together with the observations that Glis3 is expressed early in pancreatic development and that the reduction in the proendocrine compartment in *Glis3*$^{zf/zf}$ mutant mice was detectable as early as E13.5, suggests that Glis3 plays a major role in regulating the generation rather than the survival of the β-cell population.

Early multipotent pancreatic progenitors, marked by Pdx1, Ptf1a, Nkx2.2, Sox9, and Cpa1 expression (12, 57), are the source of all differentiated cells of the exocrine, ductal, and endocrine cell lineages. Sox9 expression is restricted to a mitotically active, Notch-responsive subset of Pdx1 multipotent progenitor cells and is not expressed in committed endocrine cell precursors or differentiated cells (46). Sox9 regulates the maintenance and promotes the proliferation and survival of these multipotent progenitors. Our results show that at E13.5, the expression pattern of Sox9 in the *Glis3*$^{zf/zf}$ pancreas is not significantly changed (Fig. 7A and B). Moreover, the patterns of Pdx1 staining in the pancreata of WT and *Glis3*$^{zf/zf}$ mutant

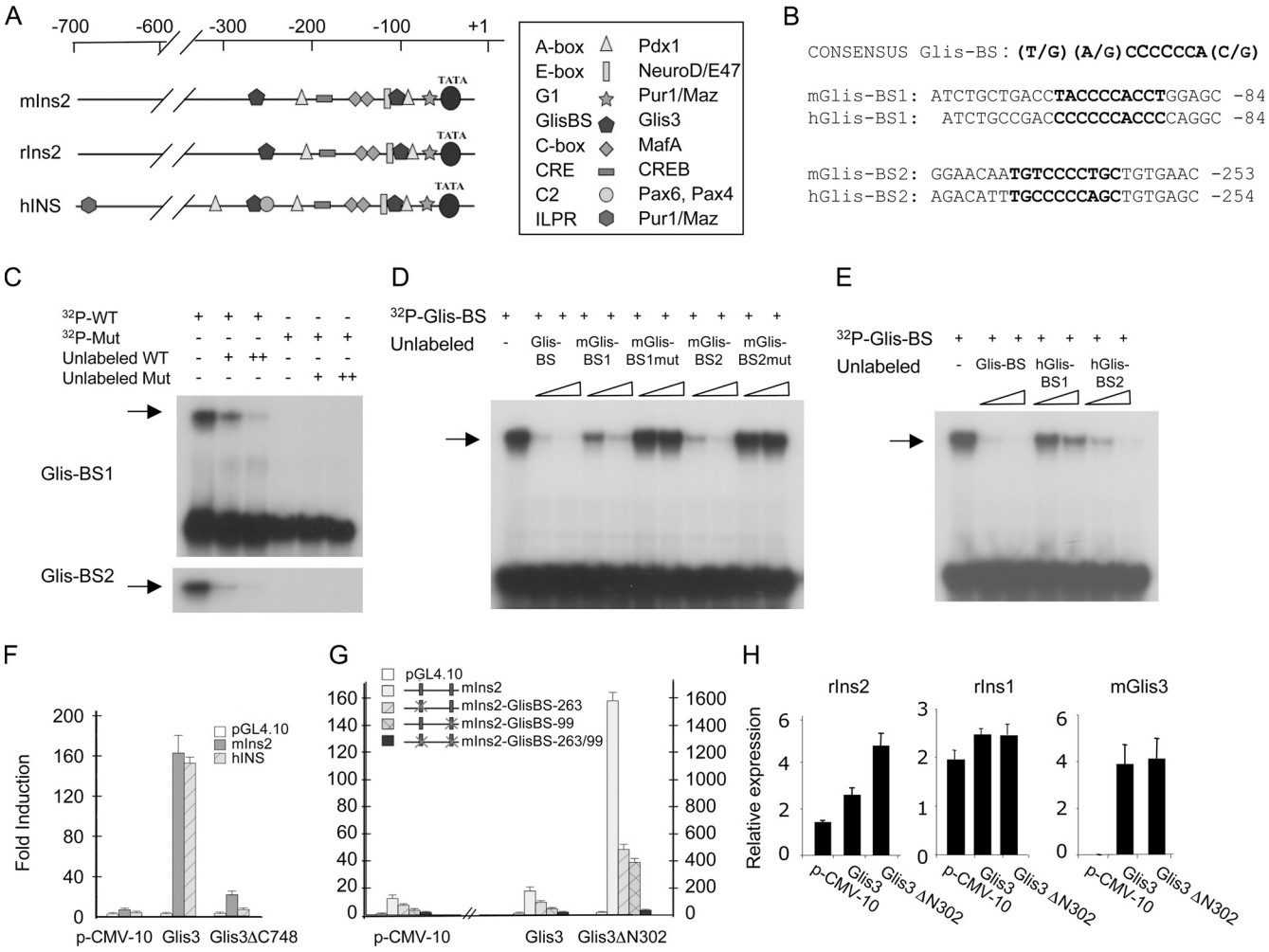

FIG. 9. Glis3 functions as a transcriptional regulator of insulin gene expression. (A) Schematic representation of potential Glis-BS and other enhancers in the murine and rat Ins2 and human INS 5′ regulatory regions. The scale at the top is in base pairs relative to the transcriptional start site (represented as +1). (B) Comparison of the nucleotide sequences of the consensus Glis-BS with the two putative Glis-BS in the mouse *Ins2* and human *INS* proximal promoter regions. (C) Two putative Glis-BS-containing regions in the mouse insulin 2 promoter (Glis-BS1 at −84 to −109 and Glis-BS2 at −253 to −276) and their respective mutant nucleotides were labeled and used as probes in an EMSA. Competition was carried out with 5-fold (+) and 25-fold (++) excesses of unlabeled oligonucleotides as indicated. Arrow indicates the Glis3-Glis-BS complex. (D, E) Binding of Glis3 to ^{32}P-labeled consensus Glis-BS was examined in the absence or presence of unlabeled consensus Glis-BS, mouse Glis-BS1, and mGlis-BS2 sites, their mutants (mut), or the corresponding human *INS* Glis-BS. (F) INS-1 (832/13) cells were cotransfected with the pGL4.10 empty vector, mIns2-696-Luc, or hINS-700-Luc and either the p3xFlag-CMV-10 empty vector, p3xFlag-CMV-Glis3, or p3xFlag-CMV-Glis3ΔC748 as indicated. After 24 h, cells were assayed for luciferase and β-galactosidase activities and the relative Luc activity was calculated and plotted. Each bar represents the mean ± the standard error of the mean. (G) INS-1 (832/13) cells were cotransfected with the pGL4.10 empty vector, mIns2(−696)-Luc, or the specified mIns2(−696)-Luc mutant and either p3xFlag-CMV-10, p3xFlag-CMV-Glis3, or p3xFlag-CMV-Glis3ΔN302 as indicated. Relative Luc activity was calculated and plotted. In the insert, shaded boxes indicate Glis-BS and X indicates mutated Glis-BS. (H) INS-1 (832/13) cells were transiently transfected with p3xFlag-CMV-Glis3 or p3xFlag-CMV-Glis3ΔN302 and 48 h later analyzed by QRT-PCR for the expression of rat Ins1 and Ins2 and mouse Glis3.

E11.5 and E13.5 embryos were not significantly different (Fig. 6B and F and 7C and D). However, the number of Pdx1$^+$ cells was dramatically reduced at E16.5 and PND3 (Fig. 7I and J and 5C and F). After E15.5, expression of Pdx1 is known to become largely associated with β cells. Thus, this decrease in Pdx1$^+$ cells may largely reflect the reduction of β-cell development in *Glis3$^{zf/zf}$* mutant mice. These observations suggest that Glis3 does not regulate pancreatic progenitor maintenance or its patterning but acts at a later stage of mouse pancreagenesis (Fig. 10). This is consistent with our observation that the size of the PND3 pancreas was not significantly changed in *Glis3$^{zf/zf}$* mutant mice; a reduction in the pancreatic progenitor pool would likely result in hypoplasia of all differentiated cell compartments.

A large number of transcription factors affect the generation of β cells (1, 18, 19, 21, 26, 28, 36). Ngn3 has been considered to be one of the key factors promoting the differentiation of pancreatic multipotent progenitors into endocrine cell progenitors (15, 17, 21, 36, 53). Ngn3 is dramatically induced at the beginning of the second transition, peaks at E15.5, and then rapidly decreases. Ngn3 expression is under complex control, which includes positive regulation by Hnf6 (20) and a Notch1/

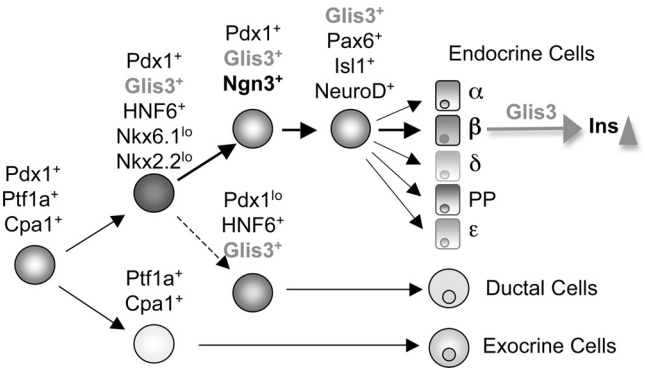

FIG. 10. Schematic representation of the critical roles of Glis3 in pancreatic development and insulin gene expression. Pdx1$^+$ Ptf1a$^+$ Cpa1$^+$ progenitor cells can differentiate into proendocrine, ductal, and exocrine lineages. Glis3 expression is induced during the second transition of pancreatic development, and Glis3 remains highly expressed in proendocrine and ductal cells and in β cells at later stages of development. Loss of Glis3 transactivation activity results in the formation of pancreatic cysts and greatly impedes the generation of β cells, which leads to the development of diabetes. In addition to regulating the generation of β cells, Glis3 also regulates the transcription of the insulin gene. These observations demonstrate that Glis3 has multiple functions in the pancreas and is a critical regulator of β-cell development and insulin expression.

Hes negative feedback loop (4, 18). Ngn3 regulates the transcription of Myt1, Insm1, Pax4, Nkx2.2, MafA, and NeuroD1, all of which play important roles in endocrine development. Analysis of Ngn3 expression shows that the number of Ngn3$^+$ cells is greatly diminished in the *Glis3$^{zf/zf}$* mutant mouse pancreas at E13.5 and E16.5. Given the important role of Ngn3 in the generation of proendocrine progenitors, our results suggest that Glis3 either regulates the transition of pancreatic progenitors to endocrine cell progenitors and/or the maintenance of endocrine cell progenitors (Fig. 10). Further studies are needed to determine the precise role of Glis3 in endocrine development, whether Glis3 directly regulates Ngn3 transcription, and whether Glis3 and Ngn3 act cooperatively in endocrine development.

Observations showing that Glis3 is highly expressed in β cells rather than other endocrine cells (45) and that the generation of β cells is affected rather than that of α cells indicate that Glis3 may have an additional role in selectively promoting the differentiation of endocrine progenitors along one or more different lineages and/or in regulating the function of mature β cells. The latter is supported by evidence showing that Glis3 also plays a role in the regulation of insulin gene expression. We identified two putative Glis-BS, Glis-BS1 and Glis-BS2, in the proximal region of the mouse *Ins2* and human INS genes that are able to bind Glis3. The Glis-BS2 site was recently reported to be functional in Glis3-mediated activation of the rat *Ins2* promoter (54). In this study, we demonstrate that optimal activation of the mouse Ins2 and human *INS* promoters by Glis3 requires both Glis-BS1 and Glis-BS2. Our results suggest that, in addition to being a critical factor in β-cell development during pancreagenesis, Glis3, along with other transcription factors, including Pdx1, Nkx6.1, Ngn3, and MafA (3, 5, 51), is implicated in maintaining β-cell function (Fig. 10).

In addition to the impairment of β-cell generation, PND3 *Glis3$^{zf/zf}$* mutant mice develop pancreatic cysts, suggesting that Glis3 plays a role in regulating the function and maintenance of ductal epithelial cells (Fig. 10). This is consistent with the observed Glis3 expression in pancreatic ducts. We previously showed that Glis3 mutant mice also develop renal cysts (24), as was also reported for NDH patients (45). Development of pancreatic and renal cysts, together with hepatic fibrosis, often coincides, suggesting a common contributing mechanism. Although the precise molecular mechanisms responsible for cyst development have yet to be established, it has become evident that dysfunction of the primary cilium and defects in cilium-associated signal transduction pathways are key factors in the etiology of cystic diseases (47, 55). We have proposed that Glis3 is part of a primary cilium-linked signaling pathway; after its activation, Glis3 translocates to the nucleus, where it regulates gene transcription (24). Thus, the development of renal and pancreatic cysts and of hepatic fibrosis in NDH patients and *Glis3$^{zf/zf}$* mutant mice appears also to be linked to a defect in a primary cilium-associated (Glis3-dependent) pathway, as has been demonstrated for other cystic diseases. However, diabetes is not commonly associated with primary cilium dysfunction, suggesting that the regulation of β-cell development by Glis3 is mediated by a different mechanism. Interestingly, several other transcription factors with important functions in pancreatic development, including Hnf6, have been implicated in cystic disease (42). Hnf6 plays a critical role in the regulation of Ngn3 and formation of endocrine progenitors (20, 42). In addition, Hnf6, by regulating Hnf1β expression, is a key factor in ductal cell specification and maturation. Loss of Hnf6 or Hnf1β results in β-cell deficiency, cyst formation within pancreatic ducts, and the development of polycystic kidney disease in mice (11, 42). However, no immediate connection was found between the expression of Hnf6 and that of Glis3. Hnf6 was not significantly altered in the pancreata of E16.5 *Glis3$^{zf/zf}$* mutant embryos (Fig. 8M and N).

In summary, our study demonstrates that mice deficient in Glis3 function develop neonatal diabetes due to impairment in the generation of β cells and insulin expression. This is associated with a significant decrease in the Ngn3$^+$ endocrine progenitor population, suggesting that Glis3 is involved in the lineage determination of endocrine progenitors into endocrine cells and β cells in particular (Fig. 10). *Glis3$^{zf/zf}$* mutant mice will provide an excellent model in which to study the molecular mechanism underlying the development of β cells and the role of Glis3 in neonatal diabetes.

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