Pdx1 inactivation restricted to the intestinal epithelium in mice alters duodenal gene expression in enterocytes and enteroendocrine cells

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Chen C, Fang R, Davis C, Maravelias C, Sibley E. Pdx1 inactivation restricted to the intestinal epithelium in mice alters duodenal gene expression in enterocytes and enteroendocrine cells. Am J Physiol Gastrointest Liver Physiol 297: G1126–G1137, 2009.—Null mutant mice lacking the transcription factor pancreatic and duodenal homeobox 1 (Pdx1) are anaplastic and survive only a few days after birth. The role of Pdx1 in regulating intestinal gene expression has therefore yet to be determined in viable mice with normal pancreatic development. We hypothesized that conditional inactivation of Pdx1 restricted to the intestinal epithelium would alter intestinal gene expression and cell differentiation. Pdx1flox/flox;VilCre mice with intestine-specific Pdx1 inactivation were generated by crossing a transgenic mouse strain expressing Cre recombinase, driven by a mouse villin 1 gene promoter fragment, with a mutant mouse strain homozygous for loxP site-flanked Pdx1. Pdx1 protein is undetectable in all epithelial cells in the intestinal epithelium of Pdx1flox/flox;VilCre mice. Goblet cell number and mRNA abundance for mucin 3 and mucin 13 genes in the proximal small intestine are reduced in the proximal small intestine of adult mice. Goblet cell number and mRNA abundance for mucin 3 and mucin 13 genes in the proximal small intestine are significantly reduced in the proximal small intestine. Conditional Pdx1 inactivation attenuates intestinal alkaline phosphatase (IAP) activity in the duodenal epithelium, consistent with an average 91% decrease in expression of the mouse enterocyte IAP gene, alkaline phosphatase 3 (a novel Pdx1 target candidate), in the proximal small intestine following Pdx1 inactivation. We conclude that Pdx1 is necessary for maintaining appropriate gene expression in enterocytes and enteroendocrine cells of the proximal small intestine.

proximal small intestine; anterior-posterior patterning; tissue specific; conditional knockout

PANCREATIC AND DUODENAL HOMEBOX 1 (Pdx1; also known as IPF1, IDX-1, STF-1, and IUF1) is a homeodomain-containing transcription factor required for pancreas development and maintenance of islet function (1, 23, 26, 36, 41). The gene encoding Pdx1 (Pdx1) belongs to a developmentally important mammalian gene cluster, Parahox, located on mouse chromosome 5 outside the classic Hox (homeobox) cluster of genes (7). Pdx1 is expressed in the developing pancreas, stomach, and duodenum. Mice homozygous for Pdx1 null mutation (Pdx1−/−) fail to form a pancreas and die in the neonatal period within a week of birth (23, 41). Pdx1 is a known regulator of several genes essential for maintaining pancreatic cell identity and function, including insulin (42), glucose transporter 2 (58), glucokinase (60), islet amyloid polypeptide (6, 9, 53), and somatostatin (Sst) (26, 36). Both activator (2, 6, 9, 12, 17, 26, 29, 36, 42, 43, 45, 53, 55, 56, 58, 60) and repressor (3, 10, 19, 48, 51, 59) regulation of target genes by Pdx1 have been described.

In the intestinal tract, Pdx1 is maximally expressed in the most anterior duodenal region with decreased expression in the distal small intestine (18). In Pdx1−/− null mutant embryos and pups, dilated cystic malformations at the stomach/duodenum junction, absence of Brunner’s glands, areas of glucose transporter 2-positive cuboidal epithelium resembling bile duct epithelium, and reduced numbers of enteroendocrine cells have been described in the rostral duodenum (41). This initial report supports a role for Pdx1 in patterning intestinal development. In further support of this role, mice with misexpression of Pdx1 targeted to the large intestine manifest an altered midgut-hindgut union (19). In addition, immature intestinal epithelial rat IEC-6 cells can differentiate into enteroendocrine cells in response to Pdx1 overexpression (63). The intestinal phenotype in mature mice with Pdx1 inactivation has not been studied, because the Pdx1−/− null mice die in the neonatal period due to the complications of pancreatic agenesis (23, 41). Specifically, a detailed analysis of intestinal gene expression patterns in the Pdx1−/− null mice has not been described.

Adenosine deaminase (Ada) (12), gastric inhibitory polypeptide (Gip), or somatostatin (Sst) is unaffected in the Pdx1flox/flox;VilCre mice, mRNA abundance for Gip and Sst is significantly reduced in the proximal small intestine. Conditional Pdx1 inactivation attenuates intestinal alkaline phosphatase (IAP) activity in the duodenal epithelium, consistent with an average 91% decrease in expression of the mouse enterocyte IAP gene, alkaline phosphatase 3 (a novel Pdx1 target candidate), in the proximal small intestine following Pdx1 inactivation. We conclude that Pdx1 is necessary for maintaining appropriate gene expression in enterocytes and enteroendocrine cells of the proximal small intestine.

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Pdx1 inactivation (Pdx1\textsuperscript{floxed/floxed};VilCre) were characterized with respect to enterocyte, enteroendocrine, Paneth, and goblet cell lineages. Whereas Pdx1 inactivation does not seem to affect enteroendocrine, Paneth, and goblet cell differentiation in mature intestine, a phenotype of attenuated enterocyte differentiation and altered gene expression in enterocytes and enteroendocrine cells was observed following Pdx1 inactivation. The Pdx1\textsuperscript{floxed/floxed};VilCre mouse is useful for providing further information on gene expression patterning by Pdx1 in the proximal small intestine.

### MATERIALS AND METHODS

**Mouse strains.** VilCre mice [strain name: B6.SJL-Tg(Vilcre997GumJ); stock no. 004586; JAX Mice & Services, Bar Harbor, ME] are hemizygous for a Vilin-Cre transgene expressing Cre recombinase driven by a 12.4-kb fragment of the mouse villin 1 gene promoter. This promoter fragment drives high level expression of Cre recombinase within the entire intestinal epithelium (31). Onset of the Vilin-Cre transgene expression is at 12.5 days postcoitum (dpc). The Pdx1\textsuperscript{floxed/floxed} mice (designated strain name: STCK Ipf\textsuperscript{floxed/floxed}, stock no. 000440-UNC, Mutant Mouse Regional Resource Center) are homozygous for a “floxed” Pdx1 allele in which Pdx1 exon 2 is flanked by loxP target sites for Cre recombinase (a generous gift from C. V. E. Wright, Vanderbilt University, Nashville, TN) (14). Exon 2 of Pdx1 encodes the DNA-binding homeodomain. The protocol for animal use was reviewed and approved by the Stanford University Institutional Animal Care and Use Committee.

**Genotyping.** Genomic DNA was isolated from mouse tail biopsies by proteinase K digestion followed by chloroform extraction and ethanol precipitation. The presence of the Vilin-Cre transgene was detected by PCR amplification with primers 5′-GTGGGGACAGAGAGACAAGAAAACC-3′ and 5′-ACATCTTGGGTCTGCAGG-3′; the presence of the floxed Pdx1 allele with 5′-AGGTTGGCGATGATGACCTGCAC-3′ and 5′-AGACGTGGGAGACAGTGGG-3′; and the presence of the wild-type Pdx1 allele with 5′-CCCTGGGACATGCTGCATATTG-3′ and 5′-GCCAACAACTGGCAGATTC-3′.

**Generation of mice with intestinal epithelium-specific Pdx1 inactivation.** A Cre-loxP mating strategy was used to generate mice with inactivation of Pdx1 in the intestinal epithelium. Specifically, F1 offspring of an initial VilCre × Pdx1\textsuperscript{floxed/floxed} intercross mating were genotyped to confirm the presence of both the Vilin-Cre and floxed Pdx1 alleles by PCR as described above. To generate mice homozygous for the floxed Pdx1 allele, F1 generation mice were backcrossed with the Pdx1\textsuperscript{floxed/floxed} line, and Pdx1\textsuperscript{floxed/floxed};VilCre offspring were identified by genotyping for the presence of both the Vilin-Cre and floxed Pdx1 alleles and the absence of the wild-type Pdx1 allele. The backcross breeding strategy also allowed for generation of control Pdx1\textsuperscript{floxed/floxed} littermates with absence of both the Vilin-Cre and wild-type Pdx1 alleles. Pdx1\textsuperscript{floxed/floxed};VilCre and control mice were fed a regular laboratory diet and maintained under normal conditions.

**Necropsy and histological examination.** Pdx1\textsuperscript{floxed/floxed};VilCre and control Pdx1\textsuperscript{floxed/floxed} mice 3 or 6 mo of age were euthanized by CO\textsubscript{2} asphyxiation and subjected to full gross necropsy and histological examination. Tissues were fixed in 10% neutral buffered formalin, processed routinely into paraffin, sectioned at 4 μm, stained with hematoxylin and eosin, and examined by light microscopy. Liver, kidney, heart, salivary gland, spleen, adrenal gland, lung, trachea, tongue, esophagus, stomach, duodenum, pancreas, jejunum, ileum, cecum, colon, bone, bone marrow, skeletal muscle, spinal cord, brain, eye, uterus, and ovary were examined. Sections of stomach, duodenum, and pancreas were also stained and examined using routine Periodic acid-Schiff (PAS) reaction.

**Immunohistochemistry.** Tissue sections were deparaffinized, hydrated, and prepared routinely with antigen retrieval for immunodetection of Pdx1, chromogranin A/B, lysozyme, Gip, and Sst. Detection of Pdx1, Gip, and Sst required blocking nonspecific binding with the use of biotin/avidin system reagents (Avidin/Biotin Blocking kit; Vector Labs, Burlingame, CA). Sections were then incubated with the respective primary antibody for 1 h at room temperature (for chromogranin A/B and lysozyme) or overnight at 4°C (for Pdx1, Gip, and Sst), followed by corresponding biotin-conjugated secondary antibody (Vector Labs) incubation for 30 min at room temperature. The primary antibodies and concentrations used were as follows: guinea pig anti-Pdx1 antibody at 1:10,000 (a generous gift from C. V. E. Wright, Vanderbilt University), rabbit anti-chromogranin A/B antibody at 1:200 (Abcam, Cambridge, MA), rabbit anti-lysozyme antibody at 1:1,220 (Dako North America, Carpenteria, CA), goat anti-Gip antibody at 1:2,000 (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-Sst antibody at 1:1,500 (Dako North America). Immunoperoxidase reaction was performed to visualize Pdx1, chromogranin A/B, lysozyme, Gip, and Sst as brown staining by incubating the tissue sections with avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Labs) followed by substrate diaminobenzidine. The nuclei were then counterstained with Meyer’s hematoxylin. Intestinal alkaline phosphatase (IAP) activity on deparaffinized, hydrated tissue sections was visualized as red staining using Vector Red Alkaline Phosphatase Substrate Kit I (Vector Labs) according to the manufacturer’s recommendations.

**Quantification of duodenal goblet, enteroendocrine, and Paneth cells.** Duodenal segments, harvested with the stomach to preserve the pylorus and fixed and paraffin-embedded as described above, were sectioned longitudinally. Within the proximal small intestine, extending 0.7 cm immediately inferior to the pylorus, areas preserving full structural integrity from the tip of epithelial protrusions to the outer muscular layer were identified (representative regions, see Figs. 3, A and B, and 4, A and B). From these regions, 10 full-length villus epithelial projections (extending from the villus tips to the top of crypts) were randomly selected for each mouse to determine numbers for goblet or enteroendocrine cells under the microscope. Goblet cells were identified by PAS staining. Enteroendocrine cells were identified by immunohistochemical (IHC) chromogranin A/B staining. For Paneth cell quantification, crypts located at the base of the intestinal mucosa and containing at least one cell stained positively for lysozyme were initially identified (see Fig. 6, A and B). Ten crypts similar in size were then randomly selected for each mouse to determine Paneth cell numbers microscopically. The villi and crypts selected for cell quantification were similar in size and length so that similar numbers of epithelial cells were included among duodenal sections. No obvious differences in size or length were observed for villi or crypts between Pdx1\textsuperscript{floxed/floxed};VilCre and control Pdx1\textsuperscript{floxed/floxed} mice. Cells identified as described above, were counted by a single investigator blinded to the identity of the particular mouse strains from which the duodenal sections were generated. Cell numbers were determined per villus/crypt as described by Mori-Akiyama et al. (37).

**Quantification of duodenal enteroendocrine cells containing Gip or Sst.** Duodenal segments were harvested, fixed, paraffin-embedded, and sectioned as described above. Enteroendocrine cells containing Gip or Sst on the tissue sections were visualized following IHC staining as described above. For each section, 10 images were collected at equal spacing under ×20 magnification within the first 0.7 cm of small intestine, immediately inferior to the pylorus. Gip- or Sst-positive cells in the villous epithelium were identified as enteroendocrine cells showing cytoplasmic brown immunostaining for Gip or Sst, respectively, and were counted manually. The numbers of hematoxylin counterstained nuclei present in the villous epithelium were determined using Metamorph software (version 7.0; Molecular Devices). The frequency of enteroendocrine cells producing Gip or Sst was calculated as the total number of enteroendocrine cells containing Gip or Sst in the total number of nuclei in the villous epithelium for each section.

**Intestinal RNA isolation.** Small intestine from the Pdx1\textsuperscript{floxed/floxed}, VilCre and control Pdx1\textsuperscript{floxed/floxed} mice (7 wk or 6 mo old) was harvested and divided into equal one-eighth segments (each segment ~5 cm) beginning at the pylorus and extending to the distal ileum as previ-
ously described (25). The small intestine tissue was preserved in RNAlater (Qiagen, Valencia, CA) during harvest and before processing for RNA isolation. Total RNA was extracted from the first intestinal segment distal to the pylorus, representing the proximal duodenum, using the RNaseasy Mini kit (Qiagen) according to the manufacturer’s protocol. Total RNA samples were further purified using the RNeasy MinElute Cleanup kit (Qiagen). RNA concentrations were determined by optical densitometry at 260 nm, and absence of RNA degradation was confirmed using a 2100 Bioanalyzer (Agilent Technologies).

Real-time quantitative RT-PCR. For quantitative real-time RT-PCR, cDNA was synthesized from duodenal RNA using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s recommendations. Expression levels for each specific gene were determined by TaqMan gene expression assays (Applied Biosystems) with mouse gene-specific, predesigned TaqMan primers and probe sets. Rat TaqMan gene expression assay was used for mouse Sst gene, because mouse Sst TaqMan assay was not available. PCR amplification and fluorescence data collection were performed with the ABI Prism 7900 HT sequence detection system (Applied Biosystems). Each gene was assayed in quadruplicate. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used to normalize for total RNA amount among samples, because the threshold cycle (Ct) values for Gapdh from different duodenal RNA samples fell in a close range with no indication of being regulated by Pdx1 (data not shown). Normalized Ct values for each gene (ΔCtgene) were calculated as raw Ct values for the gene (Ct gene) minus corresponding raw Ct values for Gapdh (CtGapdh): ΔCt gene = Ct gene − Ct Gapdh. A relative quantification approach was used in this study to calculate relative mRNA abundance for each gene: first, a reference ΔCt value (ΔCtreference) was calculated by averaging the ΔCt gene values of individual RNA samples from the control Pdx1flox/flox mice. The differences (ΔΔCt gene) of the ΔCt gene values relative to the ΔCtreference value were calculated as ΔΔCt gene = ΔCt gene − ΔCtreference. Relative mRNA abundance values were then calculated using the formula 2−ΔΔCt gene (30).

Statistical analysis. Sample means of mRNA abundance for a given gene were compared between 6-mo-old Pdx1flox/flox;VilCre mice (n = 4) and littermate control Pdx1flox/flox mice (n = 4) using two-tailed Student’s t-test, assuming equal variance. Average number of goblet, Paneth, or enteroendocrine cells per villus or crypt, as well as average frequency of enteroendocrine cells producing Gip or Sst, in the Paneth, or enteroendocrine cells per villus or crypt, as well as average frequency of enteroendocrine cells producing Gip or Sst, in the submucosal Brunner’s glands (Fig. 1, E and I) and J, because the Villin-Cre transgene is not active in the submucosal region of the small intestine (31). Pdx1 mRNA abundance was significantly reduced 24-fold in the first 5 cm of proximal small intestine in 6-mo-old Pdx1flox/flox;VilCre mice.
Fig. 1. Pancreatic and duodenal homeobox 1 gene (Pdx1) expression in the pancreas, stomach, and proximal small intestine of mice with intestine-specific Pdx1 inactivation (Pdx1<sup>flox/flox</sup>;VilCre) and control mice homozygous for loxP site-flanked Pdx1 (Pdx1<sup>flox/flox</sup>). Nuclear Pdx1 protein was visualized by immunohistochemical (IHC) staining (brown) of tissue sections with anti-Pdx1 antibody. Pancreas sections of control Pdx1<sup>flox/flox</sup> (A) and Pdx1<sup>flox/flox</sup>;VilCre littermate mice (B) show similar Pdx1 expression in islets (intense stain), acini, and ducts. Stomach of control Pdx1<sup>flox/flox</sup> (C) and Pdx1<sup>flox/flox</sup>;VilCre littermate mice (D) show similar Pdx1 staining in pyloric glands. Duodenum of control Pdx1<sup>flox/flox</sup> mouse (E) shows abundant IHC staining for Pdx1 protein in cells of the villi, crypts, and Brunner’s glands. Mucosal Pdx1 protein is absent in villi and crypts of the duodenum of Pdx1<sup>flox/flox</sup>;VilCre littermate mouse (F), whereas Brunner’s glands possess normal levels of Pdx1 (inset). Br, Brunner’s glands. Insets in E and F show Pdx1 protein in Brunner’s glands. Sections were counterstained (blue) with hematoxylin to visualize nuclei in which Pdx1 protein is not present. Higher magnification images G–J show Pdx1 expression in mucosal and submucosal epithelium of the duodenum in Pdx1<sup>flox/flox</sup>;VilCre and Pdx1<sup>flox/flox</sup> mice. Mucosal Pdx1 protein was detected in all epithelial cells along villi (G) and crypts (I) from control Pdx1<sup>flox/flox</sup> duodenal sections. Mucosal Pdx1 protein was not detected (as shown by blue nuclear counterstain) in the duodenal sections of littermate Pdx1<sup>flox/flox</sup>;VilCre mouse (H and J) for all epithelial cells. Nuclear Pdx1 protein in submucosal Brunner’s glands is comparable in duodenal sections of Pdx1<sup>flox/flox</sup>;VilCre (J) and control Pdx1<sup>flox/flox</sup> mice (I). Arrows in G and H indicate representative goblet cells located at the base of crypts identified microscopically by their distinct shape and orientation toward the gut lumen. Insets in G and H are higher magnification images of the indicated representative goblet cells. Arrowheads in I and J indicate representative Paneth cells located at the base of the crypts identified microscopically by the pyramid shape and the presence of granules.
mice (Fig. 2B). Intestine-specific inactivation of Pdx1 is thus maintained into adulthood.

Pdx1 inactivation is restricted to the intestinal mucosal epithelium of Pdx1<sup>flox/flox</sup>;VilCre mice. To further confirm intestine-specific conditional inactivation of Pdx1 in Pdx1<sup>flox/flox</sup>;VilCre mice, we characterized the presence of Pdx1 protein in the region of the duodenal mucosa abutting the pylorus. The 12.4-kb Villin-Cre transgene mediated Pdx1 attenuation in both villi and crypts of the intestinal epithelium, as indicated by the lack of nuclear Pdx1 protein in the duodenal epithelium (both villi and crypts) of adult Pdx1<sup>flox/flox</sup>;VilCre mice (Fig. 1, F, H, and J) compared with control Pdx1<sup>flox/flox</sup> littermates (Fig. 1, E, G, and I). Gross morphological appearance of villi and crypts in the duodenal epithelium of Pdx1<sup>flox/flox</sup>;VilCre mice revealed no obvious defects (compare Fig. 1, F, H, and J with Fig. 1, E, G, and I).

Nuclear Pdx1 protein was present in cells throughout the mucosal epithelium of the duodenum in adult Pdx1<sup>flox/flox</sup> control mice, suggesting that Pdx1 is expressed in the nuclei of enterocytes (Fig. 1, E and G) and goblet cells (Fig. 1G, inset). In contrast, Pdx1 protein was undetectable in the nuclei of mucosal epithelial cells in the duodenum of adult Pdx1<sup>flox/flox</sup>;VilCre mice (Fig. 1H). Robust nuclear Pdx1 expression was present along villi and in crypts of the duodenum in Pdx1<sup>flox/flox</sup>;VilCre control mice (Fig. 1E). In the nuclei of pyramid-shaped, granule-containing cells located at the base of crypts, compared with the other epithelial cell types, Pdx1 staining was less intense (Fig. 1I), consistent with a lower abundance of Pdx1 protein in duodenal Paneth cells of control mice. Pdx1 protein was undetectable by IHC staining in all crypt cells, including the pyramid-shaped, granule-containing cells located at the base of crypt structures in the duodenum of Pdx1<sup>flox/flox</sup>;VilCre mice, consistent with conditional Pdx1 inactivation (Fig. 1J).

As expected, no noticeable differences were observed in the pattern or level of Pdx1 expression in submucosal Brunner’s glands between Pdx1<sup>flox/flox</sup>;VilCre and control Pdx1<sup>flox/flox</sup> mice (Fig. 1, I and J; E and F, insets). Offield et al. (41) reported that Brunner’s glands appear to be absent in the perinatal Pdx1<sup>−/−</sup> embryo. However, the overall structure and apparent mucus content of Brunner’s glands in adult Pdx1<sup>flox/flox</sup>;VilCre mice appeared to be indistinguishable from that in control Pdx1<sup>flox/flox</sup> littermates, because Pdx1 was not eliminated in the submucosal Brunner’s glands (Fig. 3, A and B, insets). Although dilation of Brunner’s glands was observed in Pdx1<sup>flox/flox</sup>;VilCre mice (data not shown), this is not likely due to intestine-specific Pdx1 inactivation, because the dilated Brunner’s glands also expressed Pdx1, and similarly dilated Brunner’s glands were observed in Pdx1<sup>flox/flox</sup> control mice and other wild-type nonrelated mouse strains (data not shown).

Goblet cell number and gene expression are unaffected by Pdx1 inactivation in the proximal small intestine. To evaluate whether Pdx1 inactivation has an effect on goblet cell differentiation, we determined goblet cell number in the duodenum of adult Pdx1<sup>flox/flox</sup>;VilCre and littermate control Pdx1<sup>flox/flox</sup> mice (Fig. 3). Goblet cells were visualized by PAS staining of mucin and other glycoproteins (Fig. 3, A and B). The average number of duodenal goblet cells in adult Pdx1<sup>flox/flox</sup>;VilCre mice was similar to that in Pdx1<sup>flox/flox</sup> mice (Fig. 3C), suggesting that conditional Pdx1 inactivation has minimal effect on goblet cell differentiation in mature proximal small intestine. Boyer et al. (5) similarly described that the frequency of goblet cells appears unchanged in Pdx1<sup>−/−</sup> complete null mutant mice at postnatal day 7.

To assay for the effect of intestine-specific Pdx1 inactivation on goblet cell gene expression, we quantified mucin 3 and mucin 13 mRNA levels in the proximal small intestine of Pdx1<sup>flox/flox</sup>;VilCre and control Pdx1<sup>flox/flox</sup> mice. The mRNA abundance of mucin 3 and mucin 13 genes in adult Pdx1<sup>flox/flox</sup>;VilCre mice did not differ significantly from that in control Pdx1<sup>flox/flox</sup> mice (Table 1). The results support that inactivation of Pdx1 in the proximal small intestine of adult Pdx1<sup>flox/flox</sup>;VilCre mice has minimal effects on goblet cell differentiation and gene expression.

Expression of a subset of enteroendocrine genes is significantly reduced in the proximal small intestine of Pdx1<sup>flox/flox</sup>;VilCre mice. Pdx1 is a transactivator of the insulin and somatostatin genes in endocrine pancreas and is essential for pancreas development and maintenance of islet function (reviewed in Ref. 4). Studies of Pdx1<sup>−/−</sup> complete null embryos and neonatal mice have shown that numbers of specific enteroendocrine cells are decreased in the stomach (24) and duodenum (5, 22, 24, 41), suggesting a role for Pdx1 in enteroendocrine cell development in the gastrointestinal tract. To investigate the effect of Pdx1 inactivation on enteroendocrine cell differentiation in adult mice, we characterized enteroendocrine cell number in the duodenum of Pdx1<sup>flox/flox</sup>;VilCre and control Pdx1<sup>flox/flox</sup> mice (Fig. 4). Enteroendocrine cells were identified by IHC staining for chromogranins A and B (Fig. 4, A and B).
Chromogranins are acidic glycoproteins stored and released together with peptides and amines in neuroendocrine tissues and are markers for peptide hormone-producing endocrine cells. There was great variation in the numbers of chromogranin A/B-positive enteroendocrine cells present in the duodenum of adult Pdx1flox/flox;VilCre mice. The average number of duodenal enteroendocrine cells producing chromogranin A and B was comparable between adult Pdx1flox/flox;VilCre and control Pdx1flox/flox mice (Fig. 4C). In addition, mRNA abundance for chromogranin A and B genes in the proximal small intestine of adult Pdx1flox/flox;VilCre mice was not significantly different from that of control Pdx1flox/flox mice (Table 1).

Expression of enteroendocrine genes in the proximal small intestine of adult Pdx1flox/flox;VilCre and Pdx1flox/flox mice was examined by real-time RT-PCR (Table 1). Consistent with reports that Pdx1−/− embryos and perinatal mice possess fewer numbers of enteroendocrine cells secreting Gip or Sst in the duodenum (5, 22, 24), average mRNA abundance for Gip and Sst also was significantly reduced by 87 and 54%, respectively, in the proximal small intestine of adult Pdx1flox/flox;VilCre mice compared with control mice (Table 1). A decrease in average mRNA abundance for the enteroendocrine genes peptide YY (Pyy), gastrin (Gast), and glucagon-like peptide 1 (Gcg) also was observed in the proximal small intestine of Pdx1flox/flox;VilCre mice, although this was not statistically significant. Although the numbers of duodenal enteroendocrine cells producing secretin (Sct) and cholecystokinin (Cck) have been reported to be lower in Pdx1−/− embryos (41) and perinatal mice (5, 24), the reduction in average mRNA abundance for Sct and Cck was not statistically significant in the proximal small intestine of adult Pdx1flox/flox;VilCre mice compared with controls. Expression of neuropeptide tachykinin 1 (Tac1) and transcription factor neurogenin 3 (Neurog3) genes was similar between Pdx1flox/flox;VilCre and control Pdx1flox/flox mice in the proximal small intestine. Neurog3 is required for endocrine cell development in the intestinal epithelium (21). In summary, Pdx1 inactivation reduced intestinal expression, to varying degrees, of enteroendocrine genes Gcg, Pyy, Gast, Gip, and Sst and had minimal effects on expression of Sct, Cek, Tac1, and Neurog3. Given the significant decrease in average mRNA abundance for Gip and Sst in Pdx1flox/flox;VilCre mice, Pdx1 is likely to play an important role in regulating expression of Gip and Sst in the proximal small intestine.

To investigate whether the decrease in mRNA abundance for Gip or Sst in the proximal small intestine (Table 1) results from a decrease in the number of enteroendocrine cells producing Gip or Sst, we incubated duodenal sections from Pdx1flox/flox;VilCre and control Pdx1flox/flox mice at 3 and 6 mo of age with antibodies against Gip or Sst. Enteroendocrine cells stained positively for Gip or Sst were identified and counted respectively. Although the average number of enteroendocrine cells containing Gip was reduced 32% in Pdx1flox/flox;VilCre duodenum compared with control duodenum, the difference was not statistically significant (Fig. 5A). The average number of duodenal enteroendocrine cells containing Sst was similar between adult Pdx1flox/flox;VilCre and control Pdx1flox/flox mice (Fig. 5B). Thus Pdx1 inactivation does not seem to reduce specification of the enteroendocrine cells producing Gip or Sst in the mature intestine, but rather specifically reduces transcription of Gip and Sst in those cells.

Pdx1 inactivation has minimal effect on Paneth cell differentiation and gene expression. The effect of Pdx1 inactivation on Paneth cell differentiation was evaluated by determining the numbers of Paneth cells in the duodenum of adult Pdx1flox/flox;
Table 1. Expression of representative genes for enterocytes, goblet, enteroendocrine, and Paneth cells in proximal small intestine of Pdx1<sup>flox/flox; VilCre</sup> mice

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<thead>
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<th>Gene Name (Symbol)</th>
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<td>Goblet cell</td>
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<td>Mucin 3 (Muc3)</td>
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<tr>
<td>Mucin 13, epithelial transmembrane (Muc13)</td>
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<td>Enteroendocrine cell</td>
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<td>Defensin-related sequence cryptdin peptide (Paneth cells) (Defcr-rsl)</td>
<td>80</td>
</tr>
<tr>
<td>Matrix metalloproteinase 7 (Mmp7)</td>
<td>83</td>
</tr>
<tr>
<td>Enterocyte</td>
<td></td>
</tr>
<tr>
<td>Adenosine deaminase (Ada)</td>
<td>80</td>
</tr>
<tr>
<td>Alkaline phosphatase 3, intestine, not Mn requiring (Akp3)</td>
<td>9*</td>
</tr>
<tr>
<td>Cubilin (intrinsic factor-cobalamin receptor) (Cubn)</td>
<td>185</td>
</tr>
<tr>
<td>Cytochrome b reductase 1 (Cybrd1)</td>
<td>69</td>
</tr>
<tr>
<td>Fatty acid binding protein 1, liver (Fahp1)</td>
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</tr>
<tr>
<td>Fatty acid binding protein 2, intestinal (Fahp2)</td>
<td>103</td>
</tr>
<tr>
<td>Glutathione peroxidase 2 (Gpx2)</td>
<td>102</td>
</tr>
<tr>
<td>Lactase (Lct)</td>
<td>103</td>
</tr>
<tr>
<td>Sucrase-isomaltase (Si)</td>
<td>17</td>
</tr>
<tr>
<td>Trehalase (brush-border membrane glycoprotein) (Treh)</td>
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</tr>
<tr>
<td>Villin 1 (Vil1)</td>
<td>105</td>
</tr>
<tr>
<td>Villin 2 (Ezr)</td>
<td>106</td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>Caudal type homeobox 4 (Cdax4)</td>
<td>ND</td>
</tr>
<tr>
<td>Myosin IB (Myoib)</td>
<td>87</td>
</tr>
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</table>

RNA samples were isolated from the proximal 5 cm of small intestine of littermate control mice homozygous for loxp site-flanked Pdx1 (Pdx1<sup>flox/flox</sup>, n = 4) and mice with intestine-specific Pdx1 inactivation (Pdx1<sup>flox/flox; VilCre</sup>; n = 4) at 6 mo of age and assayed using real-time quantitative RT-PCR. %Control data represent quantification of the average mRNA abundance for a given gene in Pdx1<sup>flox/flox; VilCre</sup> proximal small intestine relative to control proximal small intestine. ND, not detectable. *P < 0.05.

VilCre and control Pdx1<sup>flox/flox</sup> mice at 3 and 6 mo of age (Fig. 6). Lysozyme is present in the granules of Paneth cells and thus a marker for identifying Paneth cells in intestinal crypts (Fig. 6, A and B). Pdx1<sup>flox/flox; VilCre</sup> and control Pdx1<sup>flox/flox</sup> mice possessed similar numbers of lysozyme-positive Paneth cells in the duodenal crypts (Fig. 6C), suggesting that Pdx1 inactivation in the intestine has little effect on Paneth cell differentiation in mature duodenum.

To investigate the effect of Pdx1 inactivation on Paneth cell gene expression in the proximal small intestine of Pdx1<sup>flox/flox; VilCre</sup> mice, we determined mRNA abundance of selected Paneth cell-related genes using real-time RT-PCR (Table 1). In mouse small intestine, matrix metalloproteinase 7 (Mmp7) proteolytically activates Paneth cell α-defensins from their inactive precursors (61). Enteric defensin-α1 (Defa1) is an antimicrobial peptide specific to Paneth cells in the small intestine. Defensin-related sequence cryptdin peptide (Defcr-rsl) is a defensin-related Paneth cell gene in mice (28). In mammals, defensins are central to the host defense properties of mucosal innate immunity (reviewed in Ref. 52). Consistent with the results showing that the numbers of Paneth cells remained similar in the duodenum between Pdx1<sup>flox/flox; VilCre</sup> and control Pdx1<sup>flox/flox</sup> mice (Fig. 6), expression of Mmp7, Defa1, and Defcr-rsl mRNA was not significantly different in the proximal small intestine between adult Pdx1<sup>flox/flox; VilCre</sup> and Pdx1<sup>flox/flox</sup> mice (Table 1).

Enterocyte gene expression in the proximal small intestine of Pdx1<sup>flox/flox; VilCre</sup> mice. To investigate the role of Pdx1 in regulating enterocyte gene expression, we assayed mRNA abundance for representative enterocyte genes encoding digestive hydrolases, oxidoreductases, cytoskeletal components, and transport proteins using real-time RT-PCR in the proximal small intestine of 6-mo-old Pdx1<sup>flox/flox; VilCre</sup> and control Pdx1<sup>flox/flox</sup> mice (Table 1). mRNA abundance for digestive hydrolases Cel, glutathione peroxidase 2 (Gpx2), cytoskeletal components villin 1 (Vil1), and villin 2 (Ezr), as well as transport proteins fatty acid-binding protein 1 (liver) and 2 (intestinal) (Fahp1 and Fahp2), was not affected significantly by Pdx1 inactivation in the proximal small intestine (Table 1). mRNA abundance for Si and transport protein cubulin (Cubn) genes was slightly increased, whereas that for cytochrome b reductase 1 (Cybrd1), Ada, and trehalase (Treh) was slightly decreased, in the proximal small intestine of Pdx1<sup>flox/flox; VilCre</sup> mice. The changes, however, were not statistically significant (Table 1). Consistent with the minor change in Ada mRNA abundance in the adult mouse proximal small intestine, the level of Ada protein in the duodenum as detected by IHC was not discernibly different between adult Pdx1<sup>flox/flox; VilCre</sup> and control Pdx1<sup>flox/flox</sup> mice (data not shown).

IAP gene expression is restricted to the gut, specifically to enterocytes in the small intestinal epithelium. Real-time RT-PCR revealed that mRNA abundance for the mouse IAP gene (Akp3) was significantly decreased (11-fold) by Pdx1 inactivation in the proximal small intestine of adult Pdx1<sup>flox/flox; VilCre</sup> mice (Table 1). This result identifies Akp3 as a novel Pdx1 target gene candidate. As negative controls, mRNA abundance was determined for myosin 1B (Myoib) and caudal type homeobox 4 (Cdax4) genes. Because Pdx1 did not appear to be expressed in intestinal muscle cells (Fig. 1E), no significant difference in Myoib mRNA abundance was observed between adult Pdx1<sup>flox/flox; VilCre</sup> and control Pdx1<sup>flox/flox</sup> mice, as expected. Cdax4 belongs to the murine caudal-like family of transcription factors including intestine-specific Cdx2 and is expressed in hindgut endoderm during murine embryogenesis (13). In the proximal small intestine of both adult Pdx1<sup>flox/flox; VilCre</sup> and control Pdx1<sup>flox/flox</sup> mice, Cdax4 mRNA was thus not expected to be detectable.

Akp3 mRNA abundance was reduced significantly by Pdx1 inactivation in the proximal small intestine (Table 1). To investigate whether Pdx1 inactivation further attenuated the activity of IAP in the enterocytes, we incubated duodenal sections from adult Pdx1<sup>flox/flox; VilCre</sup> and control Pdx1<sup>flox/flox</sup> littermate mice with an alkaline phosphatase substrate. The reaction produces a red product as the indication of IAP activity. As expected (20, 62), extensive IAP activity was observed along the brush-border membrane of duodenal epithelium in control mice (Fig. 5C). In the duodenum of adult Pdx1<sup>flox/flox; VilCre</sup> mice, however, IAP activity was attenuated along the mucosal surface (Fig. 5D). Given that Pdx1 inactiva-
viation resulted in an average 91% reduction of Akp3 mRNA abundance (Table 1), the residual IAP activity detected in Pdx1fllox/fllox;VilCre mice may result from IAP protein translated from the remaining Akp3 mRNA. Interestingly, the IAP activity attenuation appeared to have a patchy mosaic pattern on the apical surface of some villi in adult Pdx1fllox/fllox;VilCre duodenum (Fig. 5D). Specifically, within the same villus, IAP activity was completely absent from the brush-border membrane of some enterocytes but was present in that of others. This suggests that intestine-specific Pdx1 inactivation may have an effect on enterocyte differentiation and that this effect may be more profound in some stem cells/crypts than the others, given that IAP is a common marker of enterocyte differentiation and maturation. Of note, Nakano et al. (38) reported that the duodenum of Akp3−/−null mutant mice shows considerable residual alkaline phosphatase activity, and subsequently, Narsawa et al. (39) identified a novel murine IAP isozone encoded by Akp6. The remaining IAP activity observed in the duodenum of Pdx1fllox/fllox;VilCre mice may thus result from the expression of Akp6.

**DISCUSSION**

During mouse embryonic development, Pdx1 expression is first detectable at 8.5 dpc in the dorsal endoderm of the primitive gut. At 9.5 dpc, most mucosal cells of the presumptive duodenum express Pdx1, and this expression persists throughout life (18). In the present study, we have characterized mice with conditional inactivation of Pdx1 in intestinal epithelial cells of Pdx1fllox/fllox;VilCre mice. The Villin-Cre transgene previously has been shown to be expressed at 12.5 dpc. It is presumed, therefore, that Villin-Cre-mediated Pdx1 inactivation begins at 12.5 dpc in Pdx1fllox/fllox;VilCre mice. This intestine-specific Pdx1 inactivation is maintained until at least 13 mo of age, suggesting that Pdx1 is inactivated in stem/progenitor cells of the intestinal epithelium of Pdx1fllox/fllox;VilCre mice. This is further supported by the observation that Pdx1 protein is undetectable in all epithelial cells (enterocyte, goblet, enteroendocrine, and Paneth cells) in the intestinal mucosa of Pdx1fllox/fllox;VilCre mice.

Intestinal epithelium-specific Pdx1 inactivation has minimal effects on goblet, Paneth, and enteroendocrine cell numbers in adult Pdx1fllox/fllox;VilCre mice (Figs. 3, 4, 5, A and B, and 6). Pdx1 inactivation, however, results in reduced mRNA transcript abundance for the enteroendocrine genes Gip and Sst (Table 1). The absence of a significant difference in the number of enteroendocrine cells containing Gip or Sst in Pdx1fllox/fllox;VilCre compared with control mice suggests that the loss of Pdx1 reduces Gip and Sst gene expression but otherwise preserves specification of Gip and Sst cells. In neonatal Pdx1−/−complete null mice and embryos, a decrease in the number of chromogranin A/B- or Gip-producing enteroendocrine cells has been reported (5, 22). As described above, no statistically significant difference was observed when the numbers of such enteroendocrine cells were compared between adult Pdx1fllox/fllox;VilCre and control Pdx1fllox/fllox mice in the present study (Figs. 4 and 5A). Pdx1−/−null mutant embryos (41) and perinatal mice (5, 24) also have been reported to possess lower numbers of duodenal enteroendocrine cells producing Sct and Cck. Although we have not investigated the effect of intestine-specific Pdx1 inactivation on specification of Stromal Cell Derived Factor 1 (SDF-1) gene expression in villus epithelial cells of Pdx1fllox/fllox;VilCre mice. This is further supported by the observation that Pdx1 protein is undetectable in all epithelial cells (enterocyte, goblet, enteroendocrine, and Paneth cells) in the intestinal mucosa of Pdx1fllox/fllox;VilCre mice.

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Sct or Cck cells in the duodenum of Pdx1<sup>flox/flox</sup>;VilCre mice, our results demonstrate that the average mRNA abundance for Sct and Cck is not significantly decreased in the first 5 cm of small intestine of Pdx1<sup>flox/flox</sup>;VilCre mice compared with controls (Table 1). Mice examined in the present study were 3–6 mo old, ages when crypts have developed and an orderly adult clonal pattern has been established for cell renewal in the small intestinal epithelium (50). By contrast, the previous reports (5, 22, 24, 41) investigating enteroendocrine cell differentiation in Pdx1<sup>−/−</sup> homozygous null mice examined neonates <2 wk old, a time when crypts are still developing and the orderly adult cell renewal pattern has not yet been established (50). Moreover, Pdx1 expression is eliminated from the outset of embryogenesis in Pdx1<sup>−/−</sup> complete null mice that die from pancreatic agenesis in the neonatal period. The differences in age of mice examined or the time of onset of Pdx1 inactivation during embryogenesis (12.5 dpc in Pdx1<sup>fl</sup>;VilCre mice) may account for the decrease in number of chromogranin A/B-, Gip-, Cck-, and Sct-producing cells reported in neonatal Pdx1<sup>−/−</sup> mice or embryos, whereas no significant difference in chromogranin A/B cells or Chga, Chgb, Cck, or Sct mRNA abundance was observed in adult Pdx1<sup>fl/fl</sup>;VilCre mice. For instance, early Pdx1 inactivation in Pdx1<sup>−/−</sup> complete null embryo and neonatal mice functions to inhibit specification of the Gip cell lineage, whereas the later onset of Pdx1 inactivation in the adult conditional knockout Pdx1<sup>fl/fl</sup>;VilCre mice appears to preserve the Gip cell lineage but reduce Gip gene expression. In addition, since Pdx1 is expressed in an anterior-to-posterior gradient, it is likely that differences in the size and location of proximal intestine segments analyzed also may account for phenotype differences between the reports.

Consistent with Pdx1 transcriptional regulation of Gip and Sst genes through interaction with regulatory DNA sequences within the respective genes (12, 22, 26, 43), mRNA expression of enteroendocrine genes Gip and Sst is reduced significantly in the proximal small intestine of Pdx1<sup>fl/fl</sup>;VilCre mice compared with control Pdx1<sup>fl/fl</sup> mice (Table 1). These results support the utility of Pdx1<sup>fl/fl</sup>;VilCre mouse as a tool for identifying novel intestinal Pdx1 target genes.

Regulation of Sst gene expression by Pdx1 mainly has been investigated in the context of the pancreas. Two tissue-specific cis-elements, TSE<sub>T</sub> and TSE<sub>II</sub>, located within the Sst gene promoter at nt −85/−99 and nt −280/−300, respectively, stimulate activity of the promoter in pancreatic cell lines (26, 27, 57). Pdx1 recognizes and binds both cis-elements (26, 36, 42, 43). The present study is the first to demonstrate that Sst mRNA abundance is decreased significantly in vivo in mouse intestinal epithelium, where Pdx1 is inactivated (Table 1), although the number of duodenal enteroendocrine cells containing Sst is not significantly reduced (Fig. 5B). The Sst gene is expressed in δ-endocrine cells of the pancreas, neurons of the hypothalamus, D cells of the digestive tract, C cells of the thyroid gland, and sensory neurons (47). Intestinal enteroendocrine D cells containing Sst are scattered throughout the small and large intestine (11, 46). Approximately 90% of intestinal Sst is present in the mucosa, whereas 10% is present in the muscular layer (44). Given that there is evidence suggesting that the Sst gene is differentially regulated in different
Fig. 6. Paneth cell numbers remain unchanged in the absence of Pdx1. Paneth cell numbers were determined by counting lysozyme-positive cells in the duodenum of adult mice. Only cells stained positively for lysozyme (brown) with the pyramid shape appearance and located at the base of crypts were counted as Paneth cells. Sections were counterstained (blue) with hematoxylin to visualize nuclei. C: quantification of Paneth cells per crypt in the duodenum of control Pdx1flox/flox and Pdx1flox/flox;VilCre mice. Quantification is presented as average (+SD) Paneth cell number per crypt (n = 3 for Pdx1flox/flox and n = 8 for Pdx1flox/flox;VilCre from 3 independent litters).

Populations of Sst-producing cells in the stomach (16, 49), it would be of interest to investigate whether the same pancreas-specific TSE2 and TSE9 elements in the Sst gene promoter or other regulatory sequences mediate Pdx1 regulation in the proximal small intestine.

It has been reported that Pdx1 can function in vitro to repress transcription of the intestinal hydrolase genes Lct and Si (19, 59). It would be anticipated that similar Pdx1 repression in vivo would result in an increase in Lct and Si mRNA abundance in the proximal small intestine of adult Pdx1flox/flox;VilCre mice. Conditional inactivation of Pdx1 in the intestinal epithelial cells, however, did not result in a significant increase in Lct and Si mRNA levels (Table 1). Although the average Si mRNA abundance was elevated, the relative change in elevation was not great enough to reach statistical significance. Similarly, Lct mRNA abundance was not significantly altered in the proximal small intestine of adult Pdx1flox/flox;VilCre mice. It is possible that the anticipated derepression of Lct and Si was not observed due to the function of other transcription factors with Pdx1-redundant repressive effects. In addition, with respect to lactase, Lct gene transcription declines sharply after weaning in mice. Lct mRNA levels were thus physiologically repressed in the small intestine of adult Pdx1flox/flox;VilCre mice examined in the present study. The derepressive effect on Lct expression by intestine-specific Pdx1 inactivation might therefore be more pronounced in preweaned Pdx1flox/flox;VilCre mice.

A Pdx1 binding site is required for activation from a duodenum-specific enhancer located in the second intron of the human Ada gene (12). This activation is observed in rodents as the intestine completes the final maturation, forming adult crypt/villus structures 2–3 wk after birth at suckling-weaning transition (12). Although there was a 20% decrease in average Ada mRNA abundance in the first 5 cm of the small intestine in 6-mo-old Pdx1flox/flox;VilCre mice, the decrease did not reach statistical significance (Table 1). It is possible that in fully differentiated adult proximal small intestine, maintenance of Ada expression may not depend on Pdx1 in mice or that a redundant Pdx1-binding site protein is capable of mediating Ada transcription.

The present study identified the mouse IAP gene, Akp3, as a novel intestinal target candidate for Pdx1 regulation (Table 1, Fig. 5, C and D). Akp3 mRNA abundance in the proximal small intestine was reduced 91% on average by Pdx1 inactivation (Table 1). Duodenal brush-border IAP activity also was attenuated in the Pdx1flox/flox;VilCre epithelium compared with control epithelium (Fig. 5, C and D). In support of this finding, the spatial expression pattern of IAP in the rat intestine correlated with that of Pdx1, in that the highest expression levels are found in the duodenum, especially in the duodenal villi and crypts (20, 62) (Figs. 1 and 2A). Furthermore, in silico analysis using the TRANSFAC database (Professional version 12.1) identified five potential Pdx1 binding sites (5'-CATYAS-3') (54) within the first 3,000 bp upstream of the Akp3 translation start site (data not shown).

Alkaline phosphatases are dimeric enzymes catalyzing the hydrolysis of phosphomonoesters with the release of alcohol and inorganic phosphate (34). IAP is associated with the brush-border membrane of intestinal epithelial cells (20, 62); it is thought to transport dietary lipids as a component of the surfactant-like particles that surround the neutral fat droplets in the villous enterocytes during fat absorption (32, 33, 64). Moreover, fatty meals increase the levels of IAP in serum and lymph (15, 35). Mice homozygous for Akp3 null mutation showed faster body weight gain when fed a high-fat diet, as well as an increased lipid clearance, after a single oral administration of corn oil (38, 40). Together, these findings indicate that IAP is involved in regulating enterocyte lipid absorption in the intestinal mucosa. In enterocytes, Pdx1 may act on Akp3 to regulate lipid metabolism, a novel aspect of Pdx1 regulation.

In summary, Pdx1flox/flox;VilCre mice with Pdx1 specifically inactivated in the intestinal epithelium are viable and fertile, exhibit no apparent developmental abnormalities, and survive a normal life span. Intestinal epithelium-specific Pdx1 inactivation has minimal effects on goblet and Paneth cell gene expression and differentiation in adult Pdx1flox/flox;VilCre mice. Expression of a subset of enteroendocrine genes, including Gip and Sst, is decreased significantly in the proximal small intesti-
tine of adult Pdx1^floxflo^;VilCre mice, whereas duodenal enteroendocrine cells producing chromogranin A and B, Gip, or Sst remain relatively similar in number compared with control mice. mRNA abundance of the enterocyte hydrolase gene Akp3 in the proximal small intestine is reduced significantly in response to intestine-specific Pdx1 inactivation. IAP activity also is attenuated following Pdx1 conditional inactivation in the brush-border membrane of duodenal epithelium. The Pdx1^floxflo^;VilCre mouse model described in the present study has resulted in identification of Akp3 as a novel Pdx1 target gene candidate. The Pdx1^floxflo^;VilCre mouse model should provide a valuable resource in future characterization of the role of Pdx1 in patterning intestinal gene expression.

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DISCLOSURES
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