Requirement of the Tissue-Restricted Homeodomain Transcription Factor Nkx6.3 in Differentiation of Gastrin-Producing G Cells in the Stomach Antrum[▽]

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Many homeodomain transcription factors function in organogenesis and cell differentiation. The *Nkx* family illustrates these functions especially well, and the *Nkx6* subfamily controls differentiation in the central nervous system and pancreas. *Nkx6.3*, a recent addition to this subfamily, overlaps *Nkx6.1* and *Nkx6.2* in expression in the hindbrain and stomach. *Nkx6.3* transcripts localize in the epithelium of the most distal stomach region, the antrum and pylorus; expression in the adult intestine is lower and confined to the proximal duodenum. *Nkx6.3*$^{-/-}$ mice develop and grow normally, with a grossly intact stomach and duodenum. These mice show markedly reduced *gastrin* mRNA, many fewer gastrin-producing (G) cells in the stomach antrum, hypogastrinemia, and increased stomach luminal pH, with a corresponding increase in *somatostatin* mRNA levels and antral somatostatin-producing (D) cells. They express normal levels of other transcription factors required for gastric endocrine cell differentiation, Pdx1, Pax6, and Ngn3; conversely, *Ngn3*$^{-/-}$ mice, which also show reduced gastrin levels, express Nkx6.3 normally. These studies implicate Nkx6.3 as a selective regulator of G- and D-cell lineages, which are believed to derive from a common progenitor, and suggest that it operates in parallel with Ngn3.

Many homeodomain transcription factors play pivotal roles in development and cell differentiation. Among homeobox genes, the *Nkx* family is second in size to the clustered *Hox* genes, and its 18 members serve diverse functions in the development of the heart, pancreas, lungs, gastrointestinal (GI) tract, central nervous system, and skeleton (15, 27, 32, 44–46). The pervasive importance of *Nkx* genes has led to speculation about a developmental "*Nkx* code," which proposes that particular combinations of coexpressed *Nkx* genes regulate cell differentiation in many tissues (44).

Nkx genes are grouped by subfamilies according to sequence homology, and the two founding paralogs of the *Nkx6* subfamily, *Nkx6.2* (previously called *Gtx*) and *Nkx6.1*, are expressed specifically and required in the developing central nervous system and pancreas (17, 38). The proteins they encode have in common a DNA sequence preference, transcriptional repression activity, and dynamically regulated expression in the mouse embryonic hindbrain and spinal cord (28, 29, 34). Early in pancreas development, *Nkx6.1* and *Nkx6.2* are coexpressed in undifferentiated epithelium; the two genes subsequently mark distinct endocrine cell populations (9). Nkx6.1-deficient mice show a profound, cell-autonomous failure to expand β-cell precursors, without an increase in other pancreatic

endocrine cell types (40). *Nkx6.1* and *Nkx6.2* compound-null mutants carry more severe defects in the pancreas, with even fewer β cells and reduced numbers of glucagon-producing α cells (9). Thus, Nkx6 subfamily function in the pancreas centers on endocrine cell differentiation.

The sequence of *Nkx6.3*, the newest member (1, 33), is closest to that of *Drosophila Nk6*, which implies that it may be the ancestral founder of the vertebrate Nkx6 subfamily (33). Like its paralogs, Nkx6.3 contains an Engrailed homology domain, which may mediate interaction with transcriptional corepressors (29). In embryonic day 12.5 (E12.5) mouse embryos, *Nkx6.3* expression is reported to be restricted to endoderm-derived cells in the gastric antrum, pyloric sphincter, and proximal duodenum and to a subset of V2 interneurons in the caudal hindbrain (1), but it is excluded from the pancreas (1, 33). Its expression overlaps that of *Nkx6.1* in the brain and that of *Nkx6.2* in the stomach.

In rodents, a stratified squamous epithelium extends from the esophagus to line the fundus or dome of the stomach, followed by the glandular mucosa of the corpus and antrum, which carries four principal specialized cell types (14). Surface mucous (also known as pit or foveolar) cells line the lumen; H/K-ATPase-expressing parietal (oxyntic) and pepsinogen-producing chief (zymogenic) cells occupy the bulk of each glandular unit. Enteroendocrine cells are the least abundant and encompass a number of sublineages, most of which produce a single hormone (41). Ghrelin-, serotonin-, somatostatin-, and histamine-secreting cells are scattered throughout the

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glandular stomach epithelium, whereas those that secrete gastrin are confined to the distal stomach, the antral-pyloric segment (36). While much is known about the morphology and physiology of stomach epithelial cells, few transcriptional regulators that govern their differentiation have been characterized (11, 12, 21, 23, 35). Intestinal enteroendocrine differentiation is attributed to an axis of three transcription factors, Math1, Ngn3, and NeuroD (12, 48), which progressively help determine cell fates. Less is known about the transcriptional regulation of stomach endocrine cells, although Ngn3 seems to have a prominent role (12, 23). Gastrin, the principal secreted product of G cells, induces acid secretion in the stomach by stimulating enterochromaffin-like cells to release histamine, which activates parietal-cell H2 receptors to signal HCl release (47). Gene-targeting studies implicate the transcription factors Pdx1, Pax6, and Ngn3 in G-cell differentiation through mechanisms that remain unclear (12, 20, 21, 23).

To determine the in vivo requirements for Nkx6.3, we defined the gastric expression domain in detail and used homologous recombination to inactivate the mouse gene. Mutant mice lack discernible defects in gross or cellular morphology in the foregut but reveal a profound reduction in the number of gastrin-producing G cells and in levels of *gastrin* mRNA. Somatostatin-producing D cells are consistently increased in number, whereas other stomach endocrine cell lineages are unaffected. Thus, Nkx6.3 seems to function late in enteroendocrine differentiation, likely influencing the identity of G versus D cells.

MATERIALS AND METHODS

***Nkx6.3* gene targeting and genotyping.** Our targeting strategy was designed to delete exons 2 and 3, resulting in complete excision of the homeodomain. A strain 129/Sv bacterial artificial chromosome clone (Invitrogen, Carlsbad, CA) containing the *Nkx6.3* locus was digested with XbaI or KpnI to isolate 3.5- and 2.2-kb fragments corresponding to the desired 5′ and 3′ homology arms, respectively. These fragments were cloned into a pPNT-derived vector, and the targeting construct was electroporated into strain 129/Sv-derived TC1 embryonic stem (ES) cells. Clones were selected for growth in the presence of G418 and ganciclovir, and homologous recombination was identified by Southern analysis. Two recombinant clones were injected into C57BL/6 blastocysts to generate independent lines of Nkx6.3-null mice, which were maintained on a mixed C57BL/6-129/Sv genetic background.

For Southern analysis, genomic DNA was digested with EcoRI to monitor 5′ recombination and with NcoI to assess 3′ recombination. Membranes were hybridized with 283-bp to 338-bp probes derived from genomic sequences outside the homology arms (see Fig. 2A) amplified with primers AAAAGGATGATGAATCCCTTGAGGC and CCTGCTGCGAGCAGACTCAC (5′ probe) and primers GGGGTGTGGGCACGTAAAGG and CTTAGACTTAGTCTCCAAGT (3′ probe). With these restriction enzymes and probes, homologous recombination is predicted to replace a 4.0-kb band in the wild-type allele with a 6.4-kb band in the mutant allele (5′) and, at the 3′ end, a 7.3-kb band in the wild-type allele with a 3.7-kb band in the mutant allele. To distinguish alleles by PCR, three primers were used simultaneously: WTKO-F (AGGGGGTCACTACAACATGC, which corresponds to sequences 5′ of exon 2), WT-R (TTCCTCCCCACTTCCTTTCT, complementary to a fragment within exon2), and KO-R (TCGCCTTCTTGACGAGTTCT, which corresponds to a sequence in the neomycin resistance gene). WTKO-F and WT-R amplify a 0.4-kb fragment of wild-type exon 2; WTKO-F and KO-R amplify a 0.7-kb fragment in the recombined allele (see Fig. 2D).

RNA expression studies. For real-time quantitative reverse transcription (qRT)-PCR, total RNA was extracted from adult GI organs with Trizol (Invitrogen) and reverse transcribed with oligo(dT) primer and Superscript III enzyme (Invitrogen). qPCR primers, designed with the help of Primer3 software (37), were applied with SYBR green mastermix (Applied Biosystems, Foster City, CA) in an ABI Prism 7500 thermal cycler, and the results were analyzed with 7300 System Sequence Detection Software (Applied Biosystems). Results were normalized to the level of *GAPDH* mRNA.

For Northern analysis, we used a membrane carrying total RNA from multiple adult mouse tissues (Seegene, Seoul, South Korea; Fig. 1A) or one loaded with RNA extracted from adult intestine segments or distal stomach tissue with Trizol reagent (Fig. 1A and 2E). Membranes were hybridized at 65°C with a ^{32}P-labeled 700-bp probe corresponding to the 3′ coding region of mouse *Nkx6.3* and washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate.

For in situ hybridization, tissues were fixed overnight in 4% paraformaldehyde at 4°C and sections were prepared as described below for immunohistochemistry. Sections were hybridized overnight at 65°C with a digoxigenin-labeled riboprobe transcribed from the Nkx6.3 sequence cloned into Topo II (Invitrogen); constructs were confirmed by DNA sequencing. Slides were washed with 2× and 0.2× SSC, blocked with 10% fetal bovine serum, and incubated with 1:1,000 alkaline phosphatase-conjugated digoxigenin antibody (Roche, Indianapolis, IN). Staining was visualized with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (BCIP). Images were captured with an Olympus BX41 compound microscope with an attached charge-coupled device camera and processed with Photoshop 7.0 (Adobe) to match the microscopic view.

Immunohistochemistry and tissue staining. Adult mice were sacrificed between 2 and 10 months, and their GI organs were fixed overnight in 4% paraformaldehyde for in situ hybridization and some immunohistochemistry (gastrin) applications or in Bouin's solution (Sigma, St. Louis, MO) for histochemistry and other immunohistochemical applications (chromogranins, somatostatin, ghrelin, and serotonin). After dehydration in a graded ethanol series and embedding in paraffin, 4- to 6-μm-thick tissue specimens were rehydrated, treated with 0.5% hydrogen peroxide for 30 min, and blocked with 10% fetal bovine serum for 1 h at ambient temperature. The primary antibodies used were against chromogranins A and B (Abcam, Cambridge, MA), gastrin (E5 [Cure Digestive Diseases Research Center, University of California Los Angeles] and NCL-GASp [Vision Biosystems]), somatostatin (gift from R. Lechan, Tufts University), ghrelin (gift from K. Kangawa, Osaka, Japan), serotonin (ImmunoStar), and H$^+$K$^+$-ATPase (2B6; Medical and Biological Laboratories, Nagoya, Japan). Antibodies were applied overnight at 4°C at the following dilutions: chromogranins, 1:200; gastrin, 1:6,000 for E5 and 1:1,000 for NCL-GASp; somatostatin, 1:400; ghrelin, 1:25,000; serotonin, 1:10,000; H$^+$K$^+$-ATPase, 1:1,000. Secondary antibodies were biotin-conjugated anti-mouse or anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA). Reactions were completed with the Vectastain avidin-biotin-peroxidase complex staining kit (Vector) and diaminobenzidine substrate (Sigma, St. Louis, MO). Slides were examined on an Olympus BX41 compound microscope, and results were captured with a charge-coupled device camera with Photoshop 7.0 software.

Fixed, paraffin-embedded tissues were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), or Alcian blue according to standard protocols. Tissue sections (4 to 6 μm) were hydrated in a graded ethanol series and stained with Gill's H&E for 3 min and 30 s, respectively; with Alcian blue for 30 min, followed by 1 min Nuclear Fast Red counterstaining; or with PAS reagent for 10 min and 5 min, respectively, followed by counterstaining in Harris's hematoxylin for 3 min.

Cells were counted in multiple 250-μm-long (A-P axis) areas of vertically aligned tissue sections from the antrums of several adult mice. D cells in the corpus were counted in multiple vertical glands closest to the squamous forestomach, and density was expressed per high-power microscopic field (data not shown). Unpaired *t* tests were conducted with GraphPad software (http://www.graphpad.com/quickcalcs/ttest1.cfm).

Transmission electron microscopy. Adult mice (8 to 12 weeks old) were euthanized after starvation for 3 h. The esophagus and distal duodenum were clamped, and fixative (2.5% paraformaldehyde, 5% glutaraldehyde, 0.06% picric acid, 0.1 M cacodylate, 0.06% CaCl$_2$) was infused into the stomach for 2 min before these organs were isolated and fixed overnight at 4°C in the same solution. Samples were postfixed in OsO$_4$ and embedded in Epon 812. After light microscope inspection of toluidine blue-stained 0.5-μm sections, appropriate thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 1200 electron microscope at an accelerating voltage of 80 kV.

Overexpression studies. 293TD cells were cultured to semiconfluence in Dulbecco's modified Eagle medium supplemented with antibiotics and 10% fetal bovine serum and transfected overnight with 1 μg of Nkx6.3 expression plasmid or 1 μg of pCIG vector with Transfectin (Bio-Rad). Total RNA was extracted 48 h later and analyzed by qRT-PCR.

pH and serum gastrin measurements. Age-matched mice, 5 to 17 months old, were fasted overnight with access to water. To determine gastric pH, mice were anesthetized and, after ligating the gastroesophageal and gastroduodenal junc-
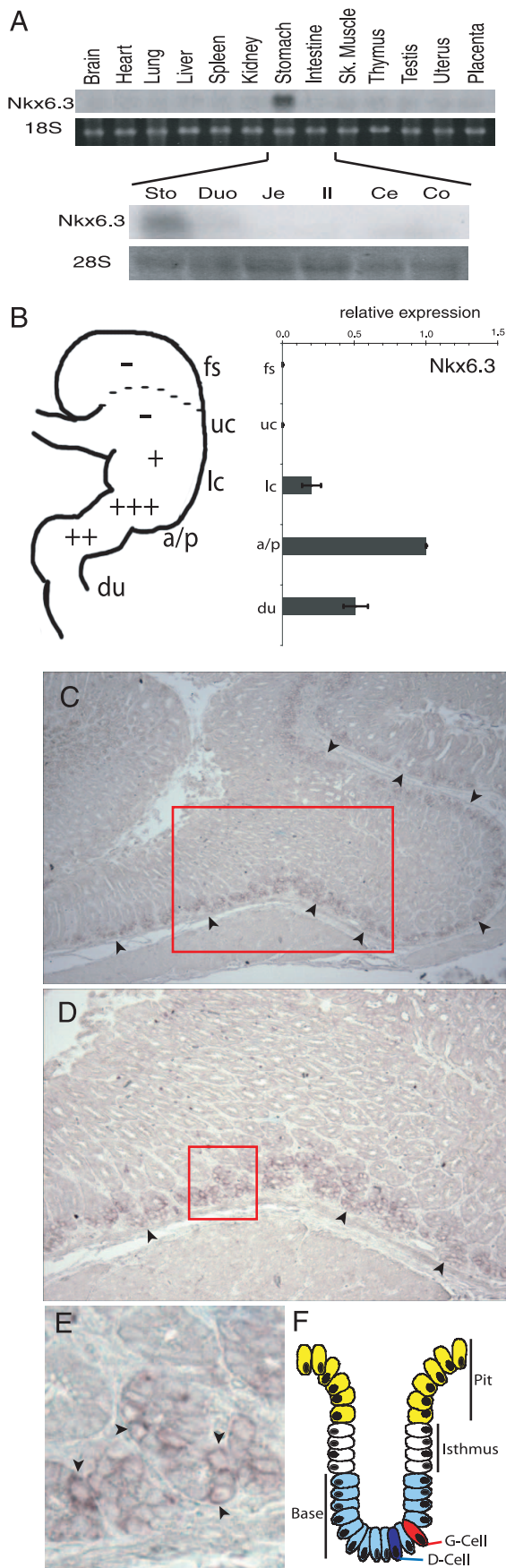
RESULTS AND DISCUSSION

Nkx6.3 expression in the GI tract. In an expression screen of ~1,400 TF genes in the developing mouse gut (3), we recognized a novel homeobox gene to be expressed much more abundantly in stomach than in the intestine. Its homeodomain sequence is most closely related to those in the Nkx6 subfamily, and the new gene is accordingly called *Nkx6.3* (1, 30, 33). Northern analysis of multiple adult mouse organs (Fig. 1A) reinforced the view that *Nkx6.3* RNA levels are especially high in the stomach and revealed its exclusion from other sites, including the brain and small intestine, organs where regionally restricted expression is evident in developing embryos (1, 33). This result may reflect reduced expression in the adult, combined with the fact that local areas of expression constitute a minor fraction of that in the whole organ. Indeed, Northern analysis of adult mouse gut segments revealed high expression in the stomach and trace levels in the duodenum but none in the jejunum, ileum, cecum, or colon (Fig. 1A).

The mouse stomach is lined by stratified squamous epithelium over the rostral one-third to one-half of its surface and by glandular mucosa over the remainder. To refine the *Nkx6.3* expression domain, we first examined discrete adult stomach regions by real-time qRT-PCR. *Nkx6.3* expression was undetectable in the squamous forestomach or upper half of the gastric corpus, and levels were highest in the antral-pyloric segment, where the mucosa has a distinctive glandular architecture; compared to the antrum, expression was fivefold lower in the distal corpus and at least twofold lower in the proximal duodenum (Fig. 1B). In situ hybridization localized *Nkx6.3*

FIG. 1. Expression of *Nkx6.3* in the stomach. (A) Northern analysis of *Nkx6.3* expression in adult mouse organs (top), showing the highest levels in the stomach, and in adult stomach and intestinal segments (bottom). The data confirm stomach (Sto) expression and reveal trace levels in the duodenum (Duo) but absence in the jejunum (Je), ileum (Il), cecum (Ce), or colon (Co). 18S or 28S RNA serves as a loading control. Sk., skeletal. (B) Relative expression of *Nkx6.3* mRNAs in isolated forestomach (fs), upper (uc) and lower (lc) corpus, antrumpylorus (a/p), and duodenum (du) samples from adult wild-type mice, as measured by qRT-PCR. All results were normalized to the level of *GAPDH* mRNA, and each segment is represented relative to an assigned wild-type antrum expression value of 1.0. The diagram depicts the anatomy and results schematically. (C) In situ hybridization on adult wild-type stomach tissue localizes *Nkx6.3* transcripts in the epithelial compartment, especially in cells at the bases of antral gland units. Arrowheads mark the continuous line of *Nkx6.3* expression along the base of the antral mucosa. (D) Higher resolution of the boxed area in panel C, showing *Nkx6.3* expression in epithelial cells at the bottoms of glands. Arrowheads point to groups of Nkx6.3-expressing cells; expression is absent in the submucosa and the upper regions of glands. (E) Magnified image of the boxed area in panel D, showing nests of epithelial cells at the gland base, bordering the submucosa. Arrowheads point to cells with the highest *Nkx6.3* signal level. (F) Graphic depiction of an antral gland unit where G and D cells occupy the base.
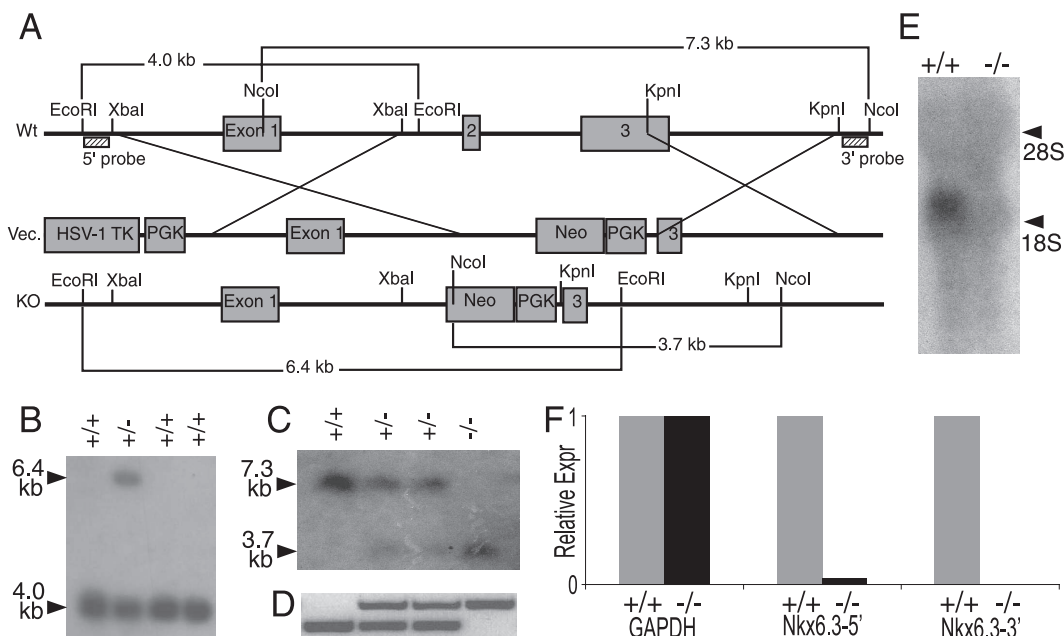
FIG. 2. Targeted disruption of the mouse *Nkx6.3* gene. (A) Gene-targeting strategy to replace exons 2 and 3 in the mouse *Nkx6.3* locus with a PGK-Neo^r cassette. Also shown are the locations of the 5′ and 3′ flanking probes used for Southern analysis and predicted band sizes in EcoRI- or NcoI-digested DNA. Wt, wild type; Vec., vector; KO, knockout. (B) Southern blot assay of ES cell DNA digested with EcoRI and interrogated with the 5′ probe. (C) Southern blot assay of mouse tail DNA digested with NcoI and interrogated with the 3′ probe. (D) PCR analysis of mouse tail DNA to distinguish *Nkx6.3* wild-type (+/+), heterozygote (+/−), and mutant (−/−) animals. (E) Northern analysis of RNA from the distal stomachs of adult wild-type and *Nkx6.3* knockout mice, demonstrating the absence of *Nkx6.3* mRNA in the latter. 18S and 28S RNA species are visible on the blot and confirmed equal loading. (F) qRT-PCR of cDNA from adult wild-type and *Nkx6.3*-null stomachs, showing that *Nkx6.3* mRNA is virtually undetectable with primers from the 5′ and 3′ ends. Expr, expression.

mRNA exclusively within the epithelial compartment of the adult antrum, near the bases of individual gastric units (Fig. 1C to F). This regional distribution in the adult stomach matches that reported in developing and newborn mice (1) and reveals Nkx6.3 as an antral marker. Furthermore, spatial restriction to the bases of individual gastric units, away from the zone of immature proliferating cells in the isthmus, suggests that only differentiated migrant cells express Nkx6.3.

These results identify Nkx6.3 as an epithelial cell product in the distal stomach, which differs from the remaining mucosa in that it lacks acid-producing (parietal) and zymogenic cells and is the exclusive residence for the endocrine G-cell population that secretes gastrin (36). Notably, *Nkx6.3* expression overlaps that of its homolog *Nkx6.2*, which is also expressed in undifferentiated pancreatic epithelium and mature insulin-producing β cells (9, 33). *Nkx6.3* expression is especially restricted and hence suggestive of specific regional functions.

Targeted disruption of the mouse *Nkx6.3* gene. To determine Nkx6.3 functions in vivo, we designed a targeting construct to delete exons 2 and 3 in ES cells, thus replacing the complete homeodomain and flanking regions with a neomycin resistance cassette (Fig. 2A). We screened G418-resistant clones by Southern analysis (Fig. 2B), identified five recombinants, and chose two clones to generate independent lines of knockout mice. The null allele was transmitted in Mendelian proportions, as judged by Southern analysis of tail DNA: with a probe from the 3′ aspect of the locus, NcoI digests revealed a 3.7-kb mutant band in place of a 7.3-kb wild-type band (Fig. 2C). PCR analysis of genomic DNA returned the expected

700-bp product corresponding to the mutant allele and a 400-bp wild-type product (Fig. 2D).

Northern analysis verified the absence of the full-length 2.1-kb RNA or other RNAs (Fig. 2E). qRT-PCR of antral stomach mRNA indicated a complete absence of transcripts from 3′ portions (exons 2 and 3) of the *Nkx6.3* gene, whereas a fragment amplified from exon 1 could be detected at <5% of wild-type levels (Fig. 2F). These data confirm the generation of a null allele and loss of the residual transcript, probably by nonsense-mediated mRNA decay.

Nkx6.3-null mice show a selective and severe reduction in G-cell numbers. Nkx6.3-null mutants gained weight at the same rate as littermates, lacked overt defects, and were fertile. Their digestive organs showed normal relationships, dimensions, and histology in both E14.5 embryos (data not shown) and 8- to 12-week-old adults (Fig. 3). As the *Nkx6.3* expression domain spans the distal stomach and proximal duodenum and overlaps that of regional transcription factor Pdx1 (20, 31), we asked first if it influences local tissue boundaries. Brünner's glands constitute a submucosal network of bicarbonate- and mucus-secreting units that are confined to the proximal duodenum (8) and resemble mucus-secreting cells found only in the distal gastric epithelium (5). We therefore tested whether Brünner's glands are a particular venue for Nkx6.3 function.

Stained tissue sections revealed a normal transition between the gastric and intestinal mucosa, with sharp demarcation (Fig. 3A and B) and no mixing between stomach glands and intestinal villi; transmission electron microscopy confirmed these impressions (data not shown). Duodenal villi showed normal
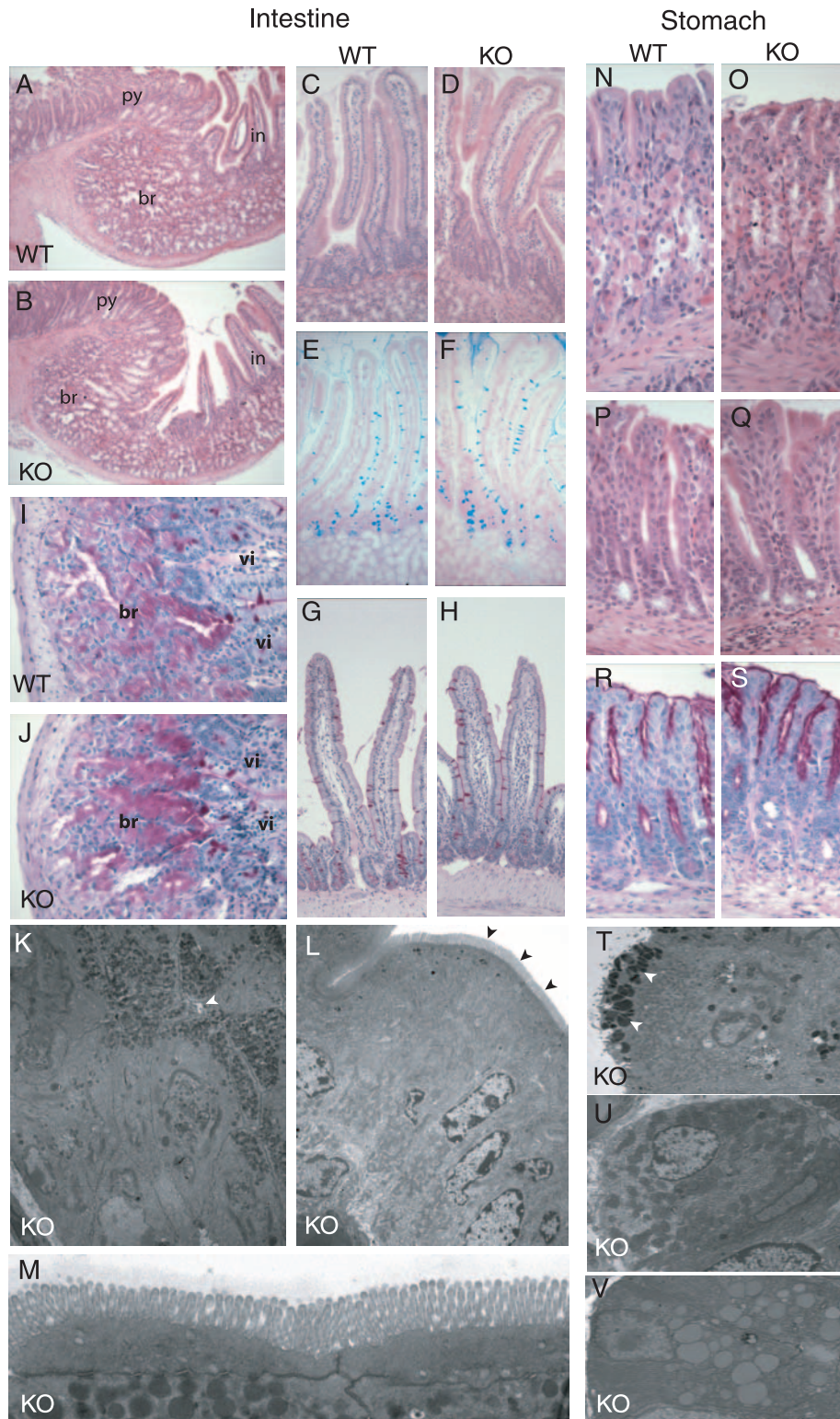
FIG. 3. Normal features of the gastro-duodenal junction, duodenum, and stomach in Nkx6.3-deficient adult (8- to 12-week-old) mice. (A, B) H&E staining at the pyloric junction of wild-type (WT) (A) and knockout (KO) (B) adult mice shows normal tissue architecture and morphology in Nkx603-null mice. py, pylorus; in, intestine, br, Brünner's glands. (C to H) H&E (C, D), Alcian blue (E, F), and PAS (G, H) staining of control (C, E, G) and knockout (D, F, H) mouse duodenums reveals normal organization and goblet cell frequency. (I, J) PAS staining demonstrates normal morphology in wild-type (I) and mutant (J) Brünner's glands. br, Brünner's glands; vi, villus. (K to M) Transmission electron micrographs of Brünner's glands cells (K, several cells facing a small central lumen pointed to by the white arrowhead), enterocytes (L), and the intestinal apical brush border (labeled with black arrowheads and magnified in panel M), revealing normal features of each compared to wild-type samples (not shown). Mucosal glands in the gastric corpus (N, O) or antrum (P to S) of the adult wild-type (N, P, R) or Nkx6.3-null (O, Q, S) stomach, stained with H&E (N to Q) or PAS (R, S) to show the normal glandular organization and dominant epithelial cell lineages. (T to V) Transmission electron micrographs of representative foveolar (T), parietal (U), and chief (V) cells in Nkx6.3-deficient mice, which show normal ultrastructural features compared to wild-type specimens (not shown). The white arrowheads in panel T point to mucin-laden granules at the apex of a foveolar (pit) cell.
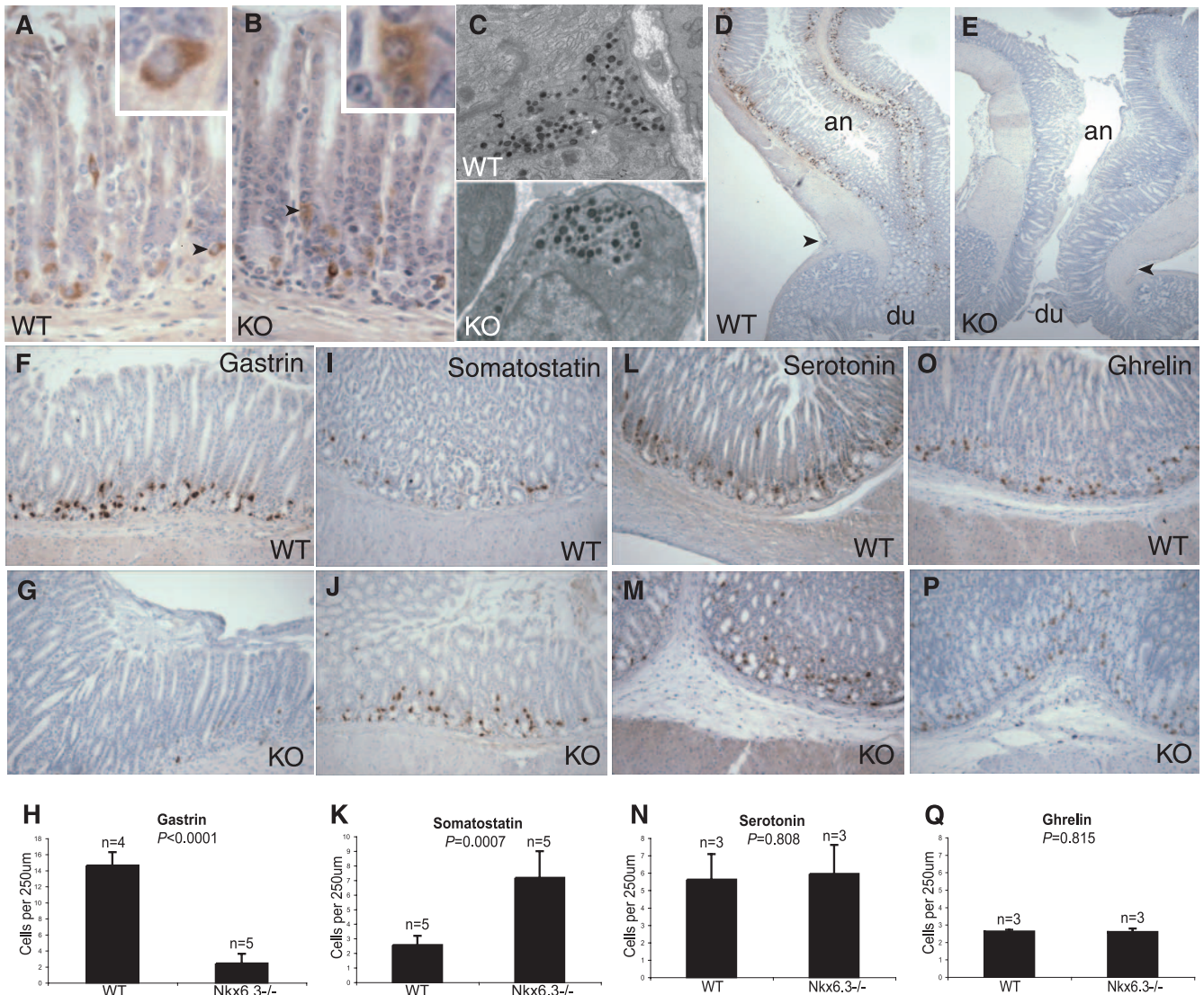
FIG. 4. Decreased number of gastric G cells in the absence of Nkx6.3. (A, B) Immunostaining for chromogranins A and B in stomach glands from wild-type (WT) (A) and knockout (KO) (B) mice. Arrowheads point to the single cells shown at a higher resolution in each inset. (C) Transmission electron micrographs of the cytoplasm, including secretory granules, of representative wild-type (top) and $Nkx6.3^{-/-}$ (bottom) gastric endocrine cells. (D to H) Gastrin immunostaining shows that the Nkx6.3-null antrum is virtually devoid of G cells, as confirmed by cell counts averaged from multiple animals (H). Low-magnification views (D, E) illustrate the antrum (an), with the duodenal (du) junction marked by arrowheads. (I to Q) Immunostaining for somatostatin (I to K), serotonin (L to N), and ghrelin (O to Q), with cell counts from identical vertical tissue sections represented in bar graphs. Photographs were taken from antral glands, and all counts were obtained in the antrum, the region with the highest $Nkx6.3$ expression level. Somatostatin-expressing D cells are increased about threefold in the $Nkx6.3^{-/-}$ antrum, whereas serotonin- and ghrelin-producing cell numbers are normal. Bar graphs represent means ± standard deviations; P values were obtained by unpaired t test. All analyses were conducted with adult mice between 8 and 12 weeks of age.

architecture (Fig. 3C and D), with normal numbers and appearance of goblet (Fig. 3E to H) and Paneth (data not shown) cells. Brünner's glands extended the same length along the duodenum in mutant and wild-type mice and were similar in organization (Fig. 3I and J) and ultrastructure (Fig. 3K). Duodenal enterocytes in $Nkx6.3^{-/-}$ adults showed wild-type morphology (Fig. 3L), with intact apical brush borders (Fig. 3M). Thus, the proximal duodenum, a site of low $Nkx6.3$ expression, seems to differentiate normally in its absence. In areas of high $Nkx6.3$ expression, the mucosa and submucosa showed normal morphology in the distal body (Fig. 3N and O) and antrum

TABLE 1. Circulating gastrin levels and stomach luminal pH in $Nkx6.3^{-/-}$ and control animals[a]

Mice	Mean serum gastrin concn (ng/liter) ± SEM (no. of mice)	P value	Mean gastric pH ± SEM (no. of mice)	P value
Wild type	87.1 ± 11.0 (7)	0.0075	3.9 ± 0.3 (16)	0.0032
Knockout	37.7 ± 10.8 (7)		5.5 ± 0.3 (7)	

[a] Values were determined in adult mice between 5 and 17 months of age that were matched for the two genotypes as described in Materials and Methods. Statistical significance was determined with the unpaired t test.
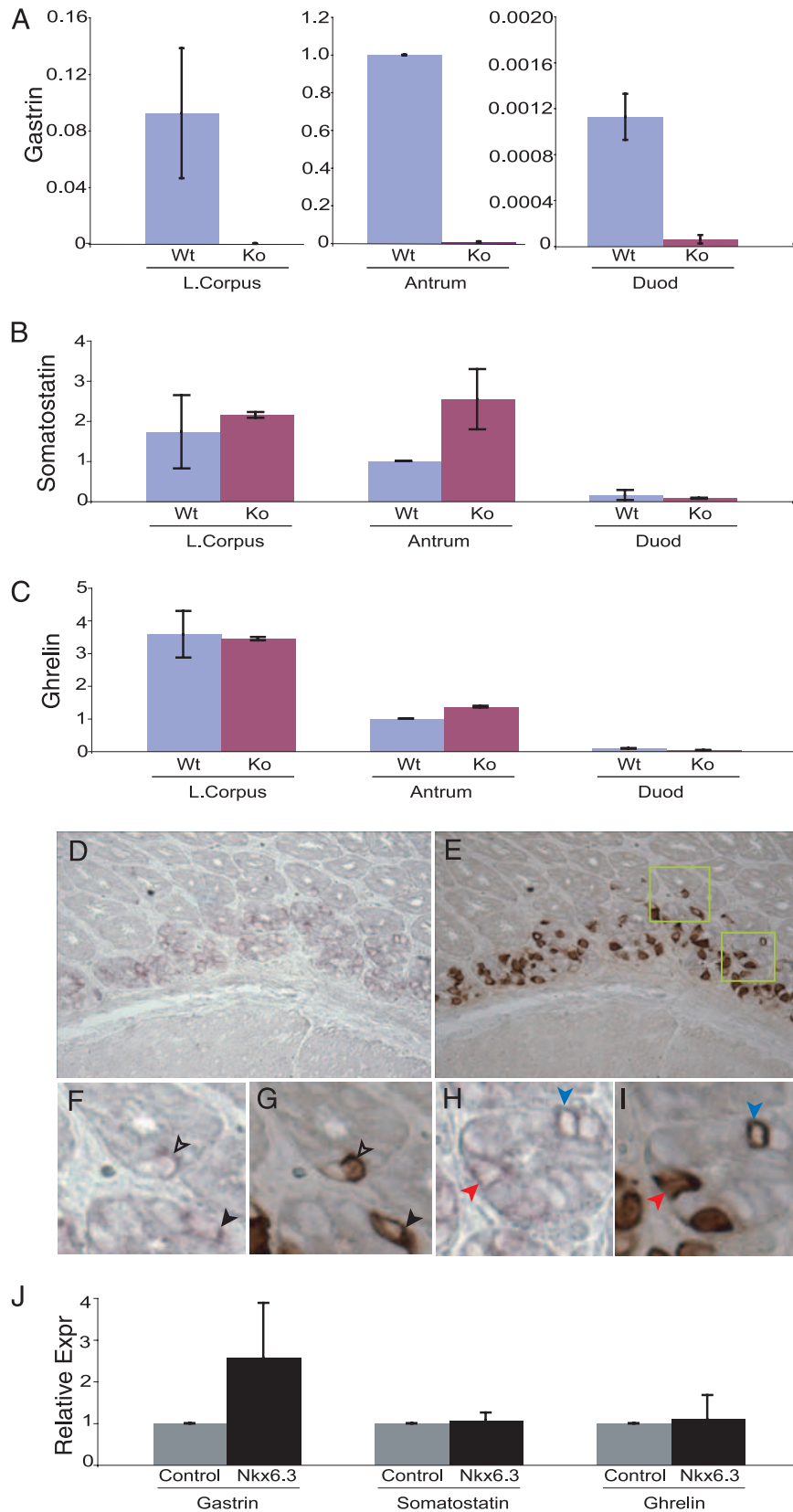
FIG. 5. *gastrin* mRNA expression is abrogated in the *Nkx6.3*-null antrum, and Nkx6.3 and gastrin colocalize in antral epithelial cells. (A to C) Relative expression of *gastrin*, *somatostatin*, and *ghrelin* mRNAs in the isolated lower (L.) corpus, antrum, and duodenum (Duod) from adult (8- to 12-week-old) wild-type (Wt) and *Nkx6.3* mutant (knockout [Ko]) mice, as measured by qRT-PCR. All results were normalized to the level of *GAPDH* mRNA, and each segment is represented relative to an assigned wild-type antral expression value of 1.0; high *gastrin* levels in the wild-type

(Fig. 3P and Q) of the mutant stomach. PAS staining indicated normal amounts and distribution of glycosylated neutral mucins (Fig. 3R and S), and H&E staining revealed the typical appearance and frequency of foveolar, parietal, and chief cells (data not shown), findings that we confirmed by transmission electron microscopy (Fig. 3T to V).

In situ hybridization places *Nkx6.3* mRNA predominantly at the bases of antral gland units (Fig. 1C to E); similar results obtained with newborn mice (1) had led another group to speculate that Nkx6.3 is expressed in chromogranin A-positive gastric endocrine cells. At first glance, we observed no anomalies in chromogranin expression or endocrine-cell ultrastructure in Nkx6.3-null mice (Fig. 4A to C). However, immunohistochemical scrutiny of individual gastric endocrine markers revealed a dramatic decrease in the number of gastrin-producing G cells in all mutant animals (Fig. 4D to H). Conversely, there was a consistent approximately threefold increase in the number of somatostatin-expressing cells in the antrum and no change in that of ghrelin- or serotonin-producing cells, which are distributed throughout the gastric glandular epithelium (Fig. 4I to Q). These results were carefully quantified by morphometric analysis of vertical tissue sections (Fig. 4H, K, N, and Q). Consistent with the reduced number of G cells, we observed a marked reduction in serum gastrin levels and a proportional elevation in the stomach luminal pH (Table 1). In contrast to *gastrin*$^{-/-}$ mice (6, 16), circulating gastrin was reduced but not absent. Nevertheless, as expected from the effects of hypogastrinemia and resulting hypochlorhydria, aged *Nkx6.3*$^{-/-}$ mice (>5 months old) showed a variable degree of parietal cell atrophy and, occasionally, a notable expansion of basal mucous cells (data not shown).

Nullizygous mice with mutations in other transcription factor genes, *Pdx1*, *Ngn3*, and *Pax6*, also have reduced G-cell numbers (12, 20, 21, 23). However, the proteins encoded by the latter genes affect additional hormonal lineages in the stomach and other organs, suggesting early or broad roles in endocrine cell differentiation. Nkx6.3 seems to have a more restricted role.

Nkx6.3 functions in G-cell differentiation are likely cell autonomous. The foregoing results suggest that Nkx6.3 is required for G-cell differentiation. In qRT-PCR analysis of the antrum, the region with the highest endogenous expression of both gastrin (25, 36) and Nkx6.3 (Fig. 1B), and the duodenum, *gastrin* mRNA was barely detectable in *Nkx6.3*$^{-/-}$ mice (Fig. 5A); it is hence unlikely that loss of gastrin immunostaining results from altered posttranscriptional events. We consistently observed ~2.5-fold higher *somatostatin* transcript levels with no change in *ghrelin* in the *Nkx6.3*$^{-/-}$ antrum (Fig. 5B and C). Alterations in *gastrin* and *somatostatin* mRNAs thus correspond to the markedly reduced G-cell and modestly increased

D-cell numbers. Although the observed changes could result from direct or indirect effects of Nkx6.3 on the expression of these genes, we suspect that the major role of Nkx6.3 is to regulate G- versus D-cell fate.

Individual endocrine lineages are defined according to the hormone they produce; there are as yet no additional accepted criteria (36), and G cells, by definition, are recognized on the basis of gastrin expression. It is formally possible that the *Nkx6.3*$^{-/-}$ mouse antrum harbors immature progenitors that fail to develop into functional G cells, but the lack of pre-G-cell markers precludes definitive resolution of this uncertainty. Candidate markers such as gastrin-releasing peptide receptor or somatostatin receptor 2 are not confined to G cells or their precursors but are expressed in many gastric mucosal cell types (2, 4).

Although gastrin and Nkx6.3 both localize at the bases of antral gland units, loss of *gastrin* gene expression in *Nkx6.3*$^{-/-}$ mice could reflect a cell-autonomous role in G cells or an effect exerted by neighboring cells. We therefore defined the *Nkx6.3* expression domain in greater detail by following *Nkx6.3* in situ hybridization with immunostaining for gastrin on the same tissue sections from wild-type adult mice. About 54% of *Nkx6.3*$^+$ cells showed gastrin expression, whereas nearly every gastrin-stained cell also expressed *Nkx6.3*; most *Nkx6.3*$^+$ *gastrin*$^-$ cells displayed a relatively weak *Nkx6.3* signal. G cells thus constitute a prominent subpopulation of *Nkx6.3*$^+$ cells, particularly those with the highest mRNA levels (Fig. 5D to I), suggesting a cell-autonomous function for Nkx6.3. To support this idea further, we expressed Nkx6.3 in 293TD kidney epithelial cells, which do not normally express gut hormones. In these heterologous cells, Nkx6.3 consistently induced modest expression of *gastrin* but not *somatostatin* or *ghrelin* transcripts (Fig. 5J), suggesting that Nkx6.3 is able to increase *gastrin* gene expression specifically. There are no data to indicate if this effect is direct or indirect.

In *gastrin*$^{-/-}$ (49), gastrin/cholecystokinin receptor-null (18), and other knockout mice with G-cell deficiency (Fig. 6A), hypochlorhydria is associated with normal or reduced D-cell numbers, often reflecting a physiologic response to sustained elevation of the gastric pH (26, 36). *Nkx6.3*$^{-/-}$ mice differ notably in that D-cell numbers increase in spite of hypogastrinemia and hypochlorhydria. Moreover, physiologic D-cell changes in response to the gastric pH appear throughout the stomach, including the corpus, which has many D (but no G) cells and lacks Nkx6.3 expression (24); gastrin infusion into *gastrin*$^{-/-}$ mice, for example, reduces D-cell density throughout the stomach (49). By contrast, *somatostatin* mRNA levels in *Nkx6.3*$^{-/-}$ mice are elevated only in the antrum and are similar to those of wild-type controls in the corpus (Fig. 5B). Corpus D-cell numbers are also similar in wild-type and mu-

antrum necessitated the use of three different *y*-axis scales in panel A. *gastrin* expression is dramatically reduced in the Nkx6.3-null antrum (A), whereas *somatostatin* expression is increased 2.5-fold (B) and *ghrelin* levels are unchanged (C). (D to I) *Nkx6.3* in situ hybridization (D, F, H) followed by gastrin immunostaining (E, G, I) on the same sections. Almost all G cells coexpress *Nkx6.3*; conversely, cells with especially high *Nkx6.3* signals coexpress gastrin, as appreciated best in high-resolution photomicrographs (F to I). Matching arrowhead colors designate *Nkx6.3* in situ hybridization (F, H) and gastrin immunostaining (G, I) on the same cells. (J) qRT-PCR analysis of *gastrin*, *somatostatin*, and *ghrelin* mRNA levels in 293TD epithelial cells in response to forced Nkx6.3 expression (Expr) by plasmid transfection. The results represent a composite of five data points from two independent experiments. All bars (A to C, J) reflect the mean ± the standard deviation.

A

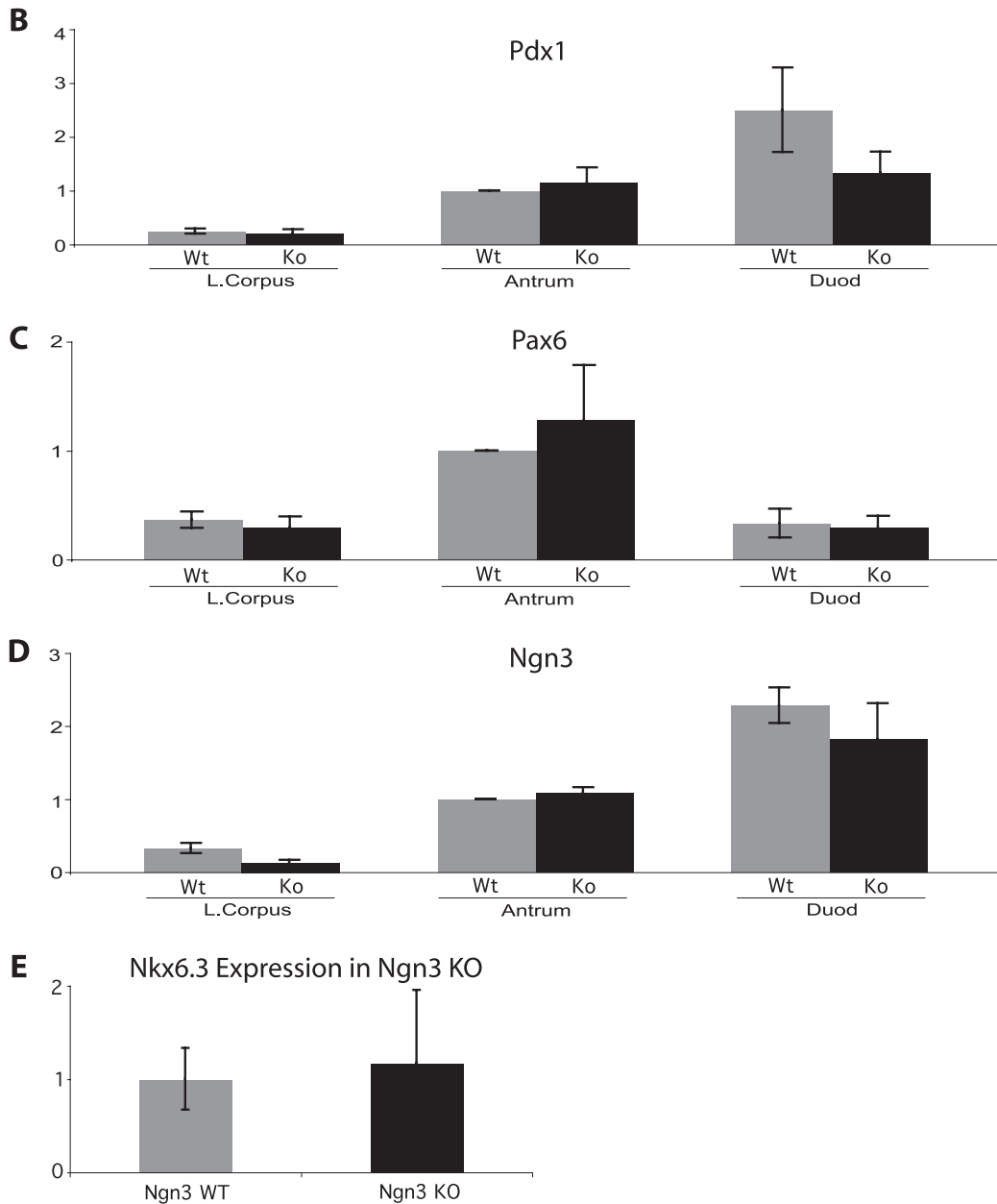| KO Strain	Ref.	Gastric Endocrine Cell Type Number in KO Mutant			
		Gastrin	Somatostatin	Serotonin	Ghrelin
Pdx1 -/-	20	Down	Unchanged	Up	
Pax6 -/-	21	Down	Down	Unchanged	
Ngn3 -/-	12	Down	Down	Unchanged	Unchanged
	23	Down	Down	Down	
CCK-B/Gastrin Receptor -/-	18	Up	Down		
Gastrin -/-	49		Unchanged		
Nkx6.3 -/-		Down	Up	Unchanged	Unchanged

FIG. 6. Nkx6.3 and Ngn3 affect G-cell differentiation through independent pathways. (A) Summary table of mutant mouse strains reported to have altered G-cell numbers and their effects on other gastric endocrine cell lineages. Empty boxes represent absence of published data. *KO*, knockout; *Ref.* reference. *Pdx1*, *Pax6*, and *Ngn3* also function in organs other than the stomach. Effects on D-cell density in mice with gastrin or its receptor knocked out are opposite to those in *Nkx6.3*$^{-/-}$ mice. (B to D) Relative expression levels of *Pdx1* (B), *Pax6* (C), and *Ngn3* (D) mRNAs are unchanged in adult *Nkx6.3*$^{-/-}$ GI segments compared to those in wild-type (Wt) mice, as measured by qRT-PCR. Results were normalized to the level of *GAPDH* mRNA, and each segment is represented relative to an assigned wild-type antrum expression value of 1.0. (E) *Nkx6.3* mRNA levels are unchanged in the neonatal Ngn3-null antrum compared to that in littermate controls. Bar graphs show the mean ± the standard deviation. L., lower; Duod, duodenum.

tant mice (43.4 ± 11 versus 45.3 ± 11 per high-power microscopic field, $P = 0.81$; data not shown). These results imply that D-cell numbers increase in the absence of Nkx6.3 by a mechanism other than a feedback effect of reduced gastrin or hypochlorhydria, and they point to a gastrin-independent effect on G-cell versus D-cell differentiation.

Nkx6.3 and antral G-cell differentiation. Larsson and colleagues have postulated that asymmetric division of bipotential G/D precursors in the isthmus of gastric glands generates gastrin- and somatostatin-producing daughter cells that remain in physical contact (19, 22). Genetic analyses have led to related inferences for other cell types; for example, $Ngn3^{-/-}$ mice have excess goblet cells and lack all endocrine lineages (12), whereas $Math1^{-/-}$ and $Hes1^{-/-}$ intestines have complementary phenotypes with respect to enterocyte and secretory-cell numbers (13, 48). Thus, multipotent gut epithelial progenitors readily adopt alternative fates when the absence of a lineage-restricted transcription factor prohibits certain choices. Our results are consistent with the notion of bipotential G/D-cell progenitors and hint that Nkx6.3 expression levels may determine G-cell identity in part. In one possible model, undifferentiated endocrine precursors in the gastric antrum require a threshold level of Nkx6.3 to differentiate into G cells, whereas those with low Nkx6.3 expression become D cells. Loss of Nkx6.3 would thus have opposite effects on gastrin- and somatostatin-producing cells, as we observed. The presence of $Nkx6.3$ mRNA in mature G cells (Fig. 5D to I) but not the isthmus of gastric glands (Fig. 1C and D) may further indicate that its expression is activated in the course of differentiation and is absent from early progenitors. Cells expressing the mutant $Nkx6.3$ gene may represent such immature cells, but the abundance of this transcript is low (Fig. 2F), either because it is unstable or because there are few progenitors. In the future, recombination-based cell lineage tracing in crosses between ROSA26 reporter (43) and $Nkx6.3^{Cre}$ mice could help establish and refine the proposed model.

Gastrin and somatostatin have opposing effects on stomach acid release: gastrin induces HCl secretion, while somatostatin inhibits it (10, 39, 42). In pathological or drug-induced states of sustained hypochlorhydria, G-cell numbers increase at the expense of D cells (36). Although molecular mechanisms of G- and D-cell differentiation are poorly understood, our results raise the possibility that antral endocrine progenitors may respond to physiologic needs to alter G/D-cell proportions by modulating cellular Nkx6.3 levels.

Nkx6.3 functions in G-cell differentiation appear to be independent of other known transcription factors. Previous studies implicate Ngn3, Pdx1, and Pax6 as transcriptional regulators of G-cell differentiation (12, 20, 21, 23), although additional endocrine defects in mice lacking these factors are distinct from those we observed in the absence of Nkx6.3 (Fig. 6A). Nevertheless, the partial overlap of endocrine phenotypes suggests that Nkx6.3 could function in a pathway shared with other G-cell factors, and we determined mRNA expression levels in Nkx6.3-null stomach tissue by qRT-PCR. There was no statistically significant difference in $Ngn3$, $Pdx1$, or $Pax6$ mRNA levels in adult $Nkx6.3^{-/-}$ and littermate control stomachs (Fig. 6B to D), indicating that Nkx6.3 is not required for their expression. We also assessed $Nkx6.3$ expression in Ngn3-null newborns, which die within 2 days after birth (7, 23);

$Nkx6.3$ mRNA levels were similar in Ngn3-null and littermate control stomachs (Fig. 6E). These data suggest that Nkx6.3 acts independently of Ngn3; both transcription factors are necessary, but neither alone is sufficient to enable $gastrin$ gene expression and G-cell differentiation.

The difference between the narrow $Nkx6.3^{-/-}$ phenotype and the diverse endocrine effects of loss of other transcription factors (Fig. 6A) suggests that the latter operate early in endocrine cell ontogeny or independently in multiple lineages. In contrast, our results reveal Nkx6.3 as a selective regulator that localizes in mature cells at the bases of antral gland units and appears to influence G- versus D-cell identity in a cell-autonomous fashion.

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