Supplemental Figure Legends

Supplemental Figure 1. Generation of a conditional Nkx2-5 allele.

Illustration of the gene targeting approach used to produce a conditional *Nkx2-5* allele. (A) The wild type *Nkx2-5* locus contains two exons. (B) The targeting vector contains a neomycin selection cassette with flanking FRT sites in intron 1; flanking loxP sites are located between the FRT/neomycin sequences and exon 1 and outside (downstream) of exon 2. (C) After homologous recombination in ES cells ("Targeted Locus"), FLP recombinase removed the neomycin cassette, producing the *Nkx2-5* conditional allele ($Nkx2\text{-}5^{flox}$), which has loxP sites flanking exon 2 and a single FRT site in intron 1 ("Flp Recombined Locus"). In cells that express *Cre* and $Nkx2\text{-}5^{flox}$, the loxP sites recombine, and the intervening sequence (including the homeodomain-containing exon 2) is deleted ("Cre Recombined Locus"); this generates a truncated *Nkx2-5* transcript, which does not produce a stable detectable NKX2-5 peptide (see Figure 6Biii).

Supplemental Figure 2. Enteric neurons express SOX9 but not NKX2-5 or GATA3.

Immunofluorescence of WT pylorus at E16.5: (i) DAPI; (ii) peripherin; (Aiii) NKX2-5; (Biii) GATA3; (Ciii) SOX9; or (iv) merged. (A-C) Enteric neurons express SOX9 (arrowheads in Cii-Civ) but not NKX2-5 or GATA3. Stomach is left; duodenum is right; and, dorsal is top. A yellow line is placed along the peripherin positive neurons between the ICM and OLM.

Supplemental Figure 3. GATA3 positive cells contribute to the serosal lining at the pylorus.

GATA3 immunofluorescence of WT pylorus at E16.5: (i-ii) merged DAPI (blue) and GATA3 (red) images; the dorsal pylorus within the boxed region in (i) is enlarged in (ii). GATA3 is expressed in the dorsal OLM and scattered squamous type cells in the serosal layer (white arrowheads in ii). Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM.

Supplemental Figure 4. The pyloric OLM is discontinuous on the ventral side.
Immunofluorescence of WT pylorus (cross section) at E14.5: (i) DAPI; (ii) NKX2-5; (iii) αSMA; (iv) GATA3; and, (v) merged. While NKX2-5 is expressed in both OLM and ICM territories, GATA3 is exclusively expressed in the OLM, which is missing on the ventral side of the pylorus (arrowheads in ii and iv). Ventral is down and left; and, dorsal is up and right.

Supplemental Figure 5. Germline loss of Gata3 or conditional deletion of Nkx2-5 results in attenuation of the dorsal pyloric OLM.
H&E staining of (A) WT, (B) *CAGGCre-ER^{TM};Nkx2-5$^{flox/flox}$* (N25 CKO), or (C) *Gata3$^{lacZ/lacZ}$* (Gata3 KO) pylorus at E18.5. The dorsal pyloric OLM (arrowheads in A-C) is prominent in WT embryos (A) but severely attenuated or absent in *Gata3* germline null embryos (B) or after *Nkx2-5* conditional deletion (C). The remainder is pylorus is histologically normal. Stomach is left; duodenum is right; and, dorsal is top. Scale bars = 100 µm.

Supplemental Figure 6. Decreased cellular proliferation in the pyloric OLM after Nkx2-5 deletion.

Immunofluorescence of (A) WT or (B) *CAGGCre-ER^{TM};Nkx2-5$^{flox/flox}$* (N25 CKO) pylorus at (Ai-ii) E14.5 or (Aiv-vi,Bi-iii) E16.5: (i,iv) DAPI; (ii,v) BrdU; or, (iii,vi) merged. (A) Proliferative cells are scattered throughout the WT ICM and OLM. (B) After *Nkx2-5* deletion, the dorsal OLM (red dashed line in Avi,Biii) is smaller but still contains proliferative cells. (C) Quantitation of BrdU positive cells, as a percentage of total (i) OLM or (ii) ICM cell mass, demonstrates decreased proliferation in the OLM but not ICM after *Nkx2-5* deletion. Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. Scale bars represent 100 µm. Error bars represent standard error of the mean.

Supplemental Figure 7. Increased cellular apoptosis in the pyloric OLM after Nkx2-5 deletion. (A) Immunofluorescence of (i-iv) WT or (v-viii) *CAGGCre-ER^{TM};Nkx2-5$^{flox/flox}$* (N25 CKO) (after 24 hours of intraperitoneal tamoxifen injections) at E16.5: (i,v) DAPI; (ii,vi) NKX2-5; (iii,vii) CASP3; or, (iv,viii) merged. (A) After *Nkx2-5* deletion, the OLM is smaller (red dashed outline in iii,vii), and there are fewer NKX2-5 positive cells (ii,vi) but more CASP3 positive cells (white arrowheads in iii,vii). (B) Quantitation of (i) total cells, (ii) total NKX2-5 positive cells, (iii) total CASP3 positive cells, (iv) percent of NKX2-5 positive cells, and (v) percent CASP3 positive cells in the dorsal pyloric OLM of WT (white) or N25 CKO (gray) mice. After *Nkx2-5* deletion, there is a significant decrease in the total number of OLM cells, with a concomitant increase in the relative number of CASP3 positive cells, indicating that there is increased cellular apoptosis. Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. Scale bars represent 100 µm. Error bars represent standard error of the mean.

Supplemental Figure 8. Germline loss of Gata3 does not abrogate Nkx2-5 expression at E14.5.

Immunofluorescence of *Gata3*$^{lacZ/lacZ}$ pylorus at E14.5: (i) DAPI; (ii) SOX9; (iii) NKX2-5; (iv) GATA3; and, (v) merged. SOX9 and GATA3 expression is lost but NKX2-5 expression persists in the dorsal OLM territory (asterisk in v). Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. Scale bars represent 100 µm.

Supplemental Figure 9. The epithelial pyloric border is unaltered in the absence of Nkx2-5 or Gata3.

CDX2 immunohistochemistry in (i) WT, (ii) *Gata3*$^{lacZ/lacZ}$ (G3KO), or (iii) *CAGGCre-ER*^{TM};*Nkx2-5*$^{flox/flox}$ (N25 CKO) pylorus at E18.5. (i) CDX2 is expressed in intestinal but not antral epithelial cells (i.e., the epithelial pyloric border) in WT pylorus. (ii-iii) Despite concomitant pyloric muscular hypoplasia, this boundary of epithelial CDX2 expression is preserved in N25 CKO and G3KO mice.

Supplemental Tables

Supplemental Table 1. Genotyping primers and protocols.

	Nkx2-5-lacZ	Gata3-lacZ
Primer1	AGT AGG CCT CGG GCT TGA	CAG GAG TCC GCG GAC CTC C
Primer2	CAG GCT AAC GAG GAG CAA TC	CCA AGC TTG GAC TCA AAA AAC T
Primer3	GTT GCA CCA CAG ATG AAA CG	GGT GGG TCG GAG GAT ACC TCT
	Nkx2-5-flox	CaggCre-ER
Primer1	TTT CGA CGG ATT CCA CAC TAG G	GCG GTC TGG CAG TAA AAA CTA TC
Primer2	GCC TCA GCT TTT ATG AGT GCA	GTG AAA CAG CAT TGC TGT CAC TT

	Nkx2-5-lacZ	Gata3-lacZ	Nkx2-5-flox	CaggCre-ER
Primer1 (µM)	1	0.4	1	1
Primer2 (µM)	1	0.4	1	1
Primer3 (µM)	0.5	0.2	N/A	N/A
$MgCl_2$ (mM)	2	0.6	2	1.92
dNTP (mM)	0.2	0.2	0.2	0.2
Initial denaturation	5 min (94°C)	5 min (94°C)	5 min (94°C)	3 min (94°C)
Cycles	35	30	30	32
Denaturation	30 sec (94°C)	30 sec (94°C)	60 sec (94°C)	30 sec (94°C)
Annealing	60 sec (60°C)	30 sec (60°C)	45 sec (58°C)	60 sec (51.7°C)
Elongation	60 sec (72°C)	30 sec (72°C)	60 sec (72°C)	60 sec (72°C)
Final elongation	10 min (72°C)	5 min (72°C)	7 min (72°C)	2 min (72°C)
Product 1 (bp)	534 (WT)	~190 (Gata3-lacZ)	246 (WT)	120
Product 2 (bp)	938 (Nkx2-5-lacZ)	325 (WT)	~400 (flox)	N/A

Supplemental Table 2. Immunofluorescence protocols.

Antibody	Blocking Media	Primary Antibody	Secondary Antibody	Tertiary Antibody
GATA3	30 minutes; RT	Overnight; 4ºC	1 hour; RT	1 hour; RT
NKX2-5 SOX9 BrdU CASP3 peripherin	30 minutes; RT	Overnight; 4ºC	1 hour; RT	N/A
αSMA	30 minutes; RT	30 minutes; RT	N/A	N/A

RT = room temperature

Supplemental Table 3. Pyloric constriction data.

	WT (μm)	N25 CKO (μm)	Gata3 KO (μm)
Length #1	234.8	347.7	453.0
Length #2	244.2	226.3	319.1
Length #3	269.9	338.1	305.6
Length #4	210.6	-	-
Average	249.7	304.0	359.2
Standard deviation	18.1	67.5	81.5

P = 0.036 (Gata3 KO vs. WT; two-tailed)
P = 0.132 (N25 CKO vs. WT; two-tailed)

Supplemental Table 4. BrdU quantitation data.

	WT OLM			N25 CKO OLM		
	DAPI (#)	BrdU (#)	BrdU (%)	DAPI (#)	BrdU (#)	BrdU (%)
Count #1	180	38	21.1	96	18	18.8
Count #2	208	33	15.9	85	12	14.1
Count #3	170	37	21.8	84	13	15.5
Count #4	125	24	19.2	100	12	12.0
Count #5	146	29	19.9	104	11	10.6
Count #6	-	-	-	89	16	18.0
Average			19.6			14.8
Standard deviation			2.3			3.2

P = 0.011 (N25 CKO vs. WT; one-tailed)

	WT ICM			N25 CKO ICM		
	DAPI (#)	BrdU (#)	BrdU (%)	DAPI (#)	BrdU (#)	BrdU (%)
Count #1	97	21	21.6	98	12	12.2
Count #2	79	17	21.5	90	17	18.9
Count #3	86	19	22.1	80	17	21.3
Count #4	86	18	20.9	124	25	20.2
Count #5	85	19	22.4	88	14	15.9
Count #6	-	-	-	64	16	25.0
Average			21.7			18.9
Standard deviation			0.6			4.4

P = 0.196 (N25 CKO vs. WT; two-tailed)

Supplemental Table 5. CASP3 quantitation data.

	CASP3 (#)	NKX2-5 (#)	Total cells (#)	CASP3 (%)	NKX2-5 (%)
WT count #1	3	104	293	1.0	35.5
WT count #2	8	184	335	2.4	54.9
WT count #3	6	111	305	2.0	36.4
WT count #4	16	216	311	5.1	69.5
WT count #5	8	145	321	2.5	45.2
Mut count #1	5	36	153	3.3	23.5
Mut count #2	10	34	145	6.9	23.4
Mut count #3	6	31	132	4.5	23.5
Mut count #4	7	33	152	4.6	21.7
WT average	8.2	152	313	2.6	48.3
WT SEM	2.4	23.9	8.0	0.8	7.1
Mut average	7	33.5	145.5	4.8	23.0
Mut SEM	1.1	1.0	4.8	0.8	0.4

$P < 0.001$ (total cells #; N25 CKO vs. WT; two-tailed)
$P = 0.003$ (NKX2-5 #; N25 CKO vs. WT; one-tailed)
$P = 0.318$ (CASP3 #; N25 CKO vs. WT; one-tailed)
$P = 0.008$ (NKX2-5 %; N25 CKO vs. WT; one-tailed)
$P = 0.034$ (CASP3 %; N25 CKO vs. WT; one-tailed)

Supplemental Methods

Genotyping

Genomic DNA for genotyping was extracted from adult tail or embryonic tissue by proteinase K digestion with high-salt ethanol precipitation. Genotyping was performed using standard PCR methods with sequence-specific primers and agarose gel electrophoresis (see Supplemental Table 1 for specific genotyping primers and protocols).

Histology

Staged E14.5-E18.5 embryos from litters of timed-pregnant dams were dissected on ice in 1X PBS. Excised gastrointestinal tracts were fixed overnight at 4°C in 4% paraformaldehyde (PFA), embedded in paraffin, cut into 5-10 μm sections with a microtome, and adhered to Superfrost Plus glass slides (Fisher Scientific, Hampton, NH, 22-034-979).

Hematoxylin and eosin (H&E) staining

For H&E staining, standard laboratory methods were used. Briefly, sections were deparaffinized in xylene, rehydrated through decreasing alcohol concentrations, stained with hematoxylin, incubated in bluing solution, counterstained with eosin, dehydrated through increasing alcohol concentrations, and equilibrated with xylene. Glass coverslips were mounted with Permount (Fisher Scientific, SP15-100). Sections were photographed with an E800 microscope (Nikon, Tokyo, Japan) and Spot CCD camera (Diagnostic Instruments, Sterling Heights, MI).

Immunofluorescence

Sections were deparaffinized in xylene, and rehydrated through decreasing alcohol concentrations. For antigen retrieval, sections were boiled for 10 minutes either in 10 mM sodium citrate, pH 6.0, or Antigen Unmasking Solution, Citric Acid Based (Vector Laboratories, Burlingame, CA, H-3300). Sections were then blocked with 10% animal serum/0.01% Triton X-100 in 1X PBS ("blocking solution") for 30 minutes at room temperature. Primary antibodies, diluted in blocking solution, were incubated on sections (duration dependent on specific antibody, see Supplemental Table 2). Sections were washed in 1X PBS prior to incubation with appropriate secondary antibodies (diluted in blocking solution) for 30 minutes at room temperature. For signal amplification, slides were washed in 1X PBS and incubated with appropriate tertiary antibodies (diluted in blocking solution) for 30 minutes at room temperature. αSMA immunofluorescence was performed by incubating sections with Cy3-conjugated primary antibody at room temperature for 30 minutes, either independently after antigen retrieval or during incubation with secondary or tertiary antibodies. Glass coverslips were mounted with ProLong Gold Antifade Reagent with DAPI (Life Technologies, Carlsbad, CA, P-36931). Sections were photographed with an Apotome microscope (Carl Zeiss AG, Jena, Germany) and AxioCam MR camera (Carl Zeiss).

Primary antibodies used were: Cy3-conjugated, mouse monoclonal to αSMA (1:500; Sigma-Aldrich, C6198); goat polyclonal to NKX2-5 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, sc-8697); mouse monoclonal to GATA3 (1:50; Santa Cruz Biotechnology, sc-268); rabbit polyclonal to SOX9 (1:200; Millipore, Dramstadt, Germany, AB5535); rabbit monoclonal to Cleaved Caspase-3 (1:200; Cell Signaling, Danvers, MA, 9664S); rat polyclonal to BrdU (1:200; Accurate Chemical & Scientific, Westbury, NY, OBT0030G); and, rabbit polyclonal to

peripherin (1:200; Millipore, AB1530). Secondary antibodies used were: biotinylated horse anti-mouse IgG (1:200; Vector Laboratories, BA-2000); 488 donkey anti-rat IgG(1:100; Life Technologies, A-21208); 488 donkey anti-rabbit IgG (1:100; Life Technologies, A-21206); and APC-conjugated donkey anti-goat IgG (1:100; Jackson ImmunoResearch, West Grove, PA, 703-136-155). Tertiary antibodies used were: Cy3/FITC/Cy5-conjugated mouse monoclonal to biotin (1:200; Jackson ImmunoResearch, 200-162-211/200-092-211/200-172-211). See Supplemental Table 2 for details of specific immunofluorescence protocols.

CDX2 immunohistochemistry

Paraffin sections were processed as described above (see Immunofluorescence). Primary mouse monoclonal antibody to CDX2 (1:200; BioGenex, San Ramon, CA; MU392A-UC) was diluted in blocking solution and incubated on sections overnight at 4ºC. Sections were washed in 1X PBS prior to incubation with a biotinylated horse pan-specific secondary antibody (1:1000; Vector Laboratories, BA-1300) and ABC reagent (Vectastain ABC Kit; Vector Laboratories, PK-6100), each for 30 minutes at room temperature. Sections were then processed using the DAB peroxidase substrate kit (Vector Laboratories, SK-4100), counterstained with hematoxylin and/or eosin, and mounted under glass coverslips with Permount. Sections were photographed as described above (see H&E staining).

BrdU labeling

A single intraperitoneal injection of BrdU (20 μg/g body weight in 1 mL of 1X PBS; Life Technologies, B23151) was administered to timed-pregnant dams, two hours prior to sacrifice; harvested embryos were then dissected and analyzed as described above.

Immunofluorescence quantitation

Four, five, or six total sections from three independent WT and *Nkx2-5* conditional null embryos were examined by double immunofluorescence for NKX2-5 and CASP3 or BrdU, as described above (see Immunofluorescence and Supplemental Table 2). Sections were photographed using the same exposure settings for each channel (DAPI, FITC, and Cy3), and the fluorescence in each channel was depicted as a single color (red). For each section, one investigator (A.P.) used the DAPI channel to define an area for examination, denoted by a white line in all four channel images, and counted the total number of nuclei. A.P. then randomly assigned numbers to each section, blinding the genotype for another investigator (D.S. for CASP3 and another lab member for BrdU), who counted the number of discrete fluorescent signals (Supplemental Tables 4 and 5). Cell number and percentage of total cells were analyzed by the Student's t-test statistic in Excel (Microsoft, Redmond, WA; unpaired, heteroscedastic, one- or two-tailed).

Measurement of pyloric sphincter constriction

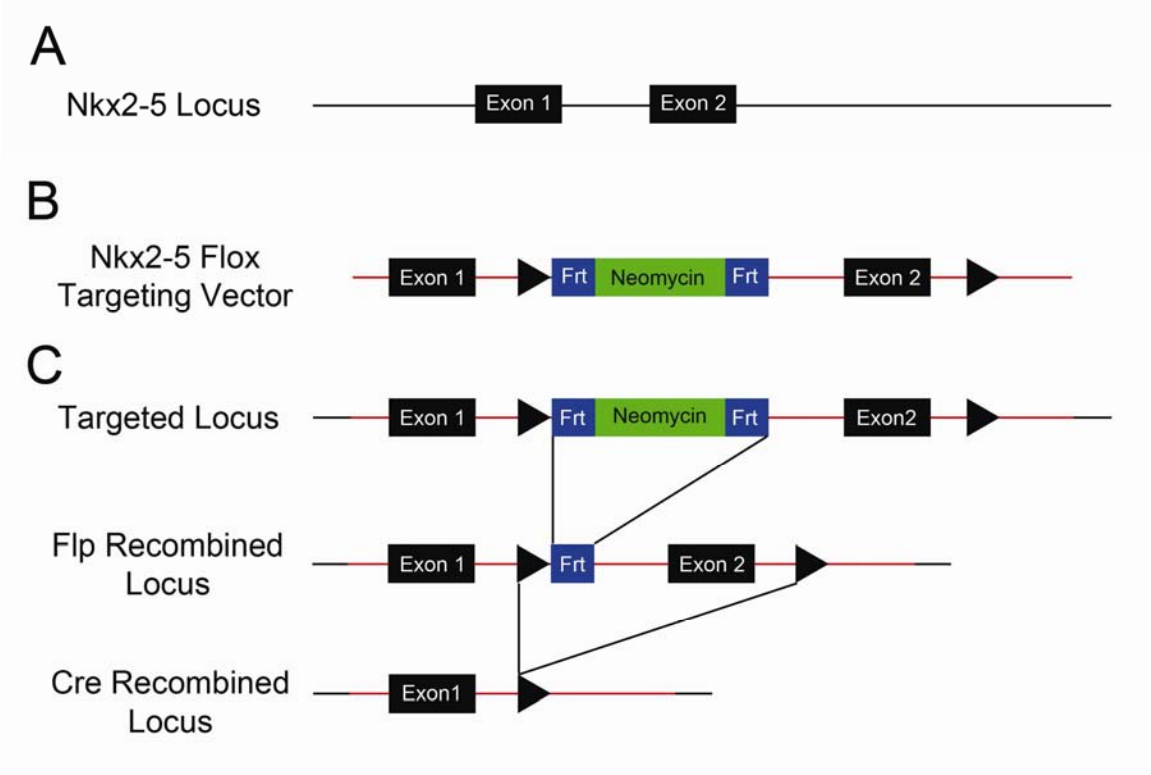
A single section from at least three independent WT, *Gata3* deficient, and *Nkx2-5* conditional null embryos was examined by immunofluorescence for αSMA, as described above (see Immunofluorescence and Supplemental Table 2). The shortest distance between the smooth muscle (ICM) layers on opposite sides of the pyloric lumen was measured with ImageJ (United States National Institutes of Health, Bethesda, MA)[1]. The measurements were averaged and analyzed by the Student's t-test statistic in Excel (as described above).

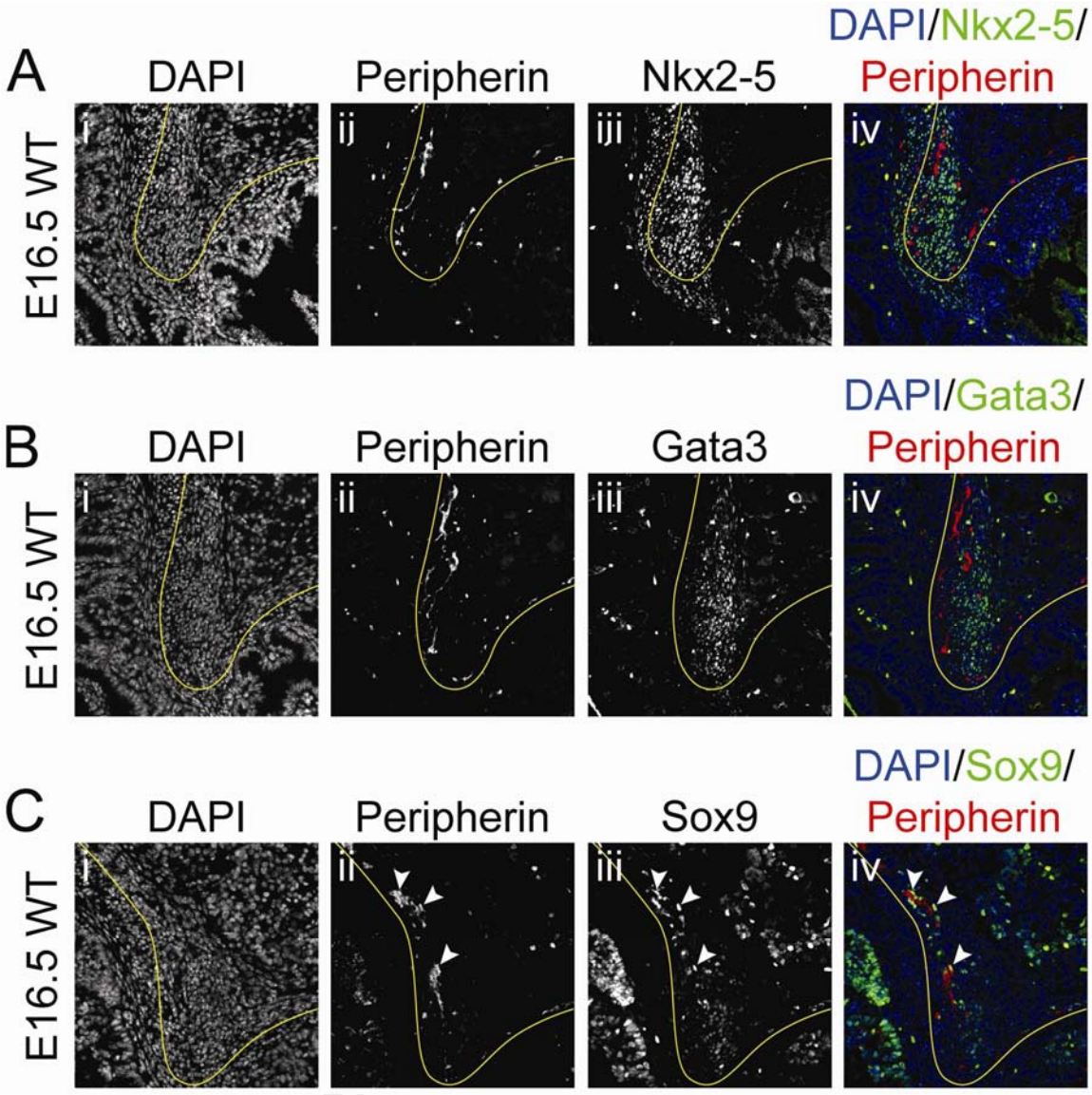
Whole mount X-gal staining

Staged *Gata3*$^{lacZ/+}$ or *Nkx2-5*$^{lacZ/+}$ embryos were dissected on ice in 1X PBS. The stomach and adjoining duodenum was excised, fixed with 4% PFA for 10 minutes at 4°C, and washed well in 1X PBS. X-gal staining solution was prepared fresh, as follows: 1X PBS (pH 7.5), 2 mM magnesium chloride, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg X-gal (from 20 mg/ml stock in DMF; Life Technologies, 15520-04). Fixed tissue was incubated in staining solution at 37°C and monitored periodically to control staining intensity. Stained tissue was washed well in 1X PBS and then post-fixed overnight at 4°C in 4% PFA. Whole mount tissue was photographed in 1X PBS or 80% glycerol (in 1X PBS) with a Leica MZ12.5 dissecting microscope (Solms, Germany) and Spot CCD camera.

Supplemental References

1. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671-5.

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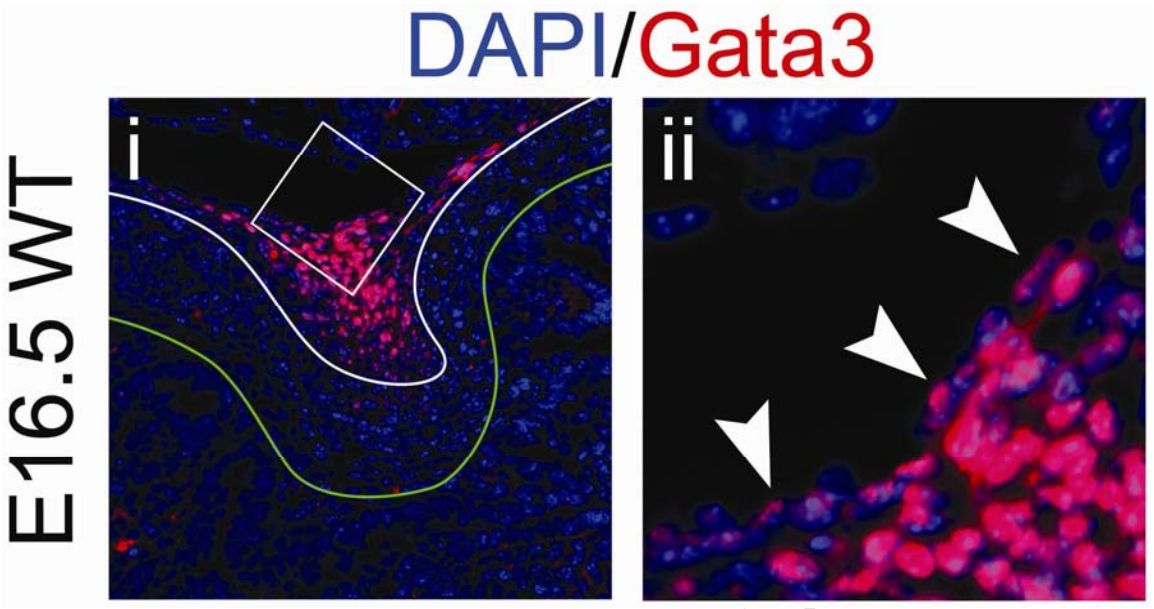

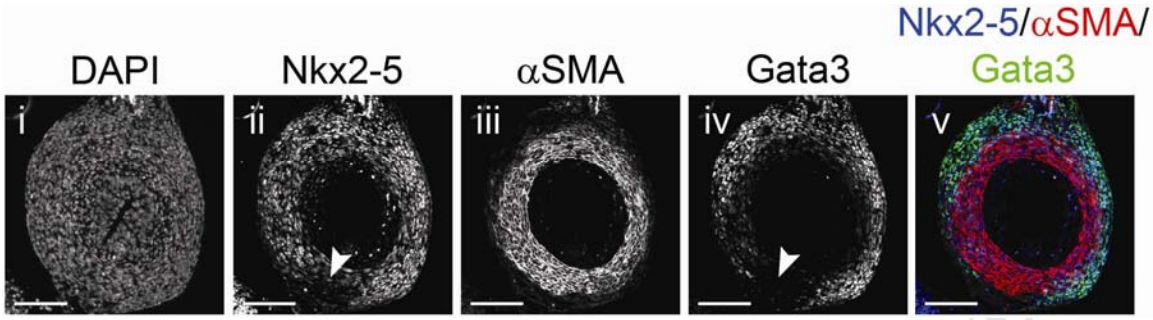

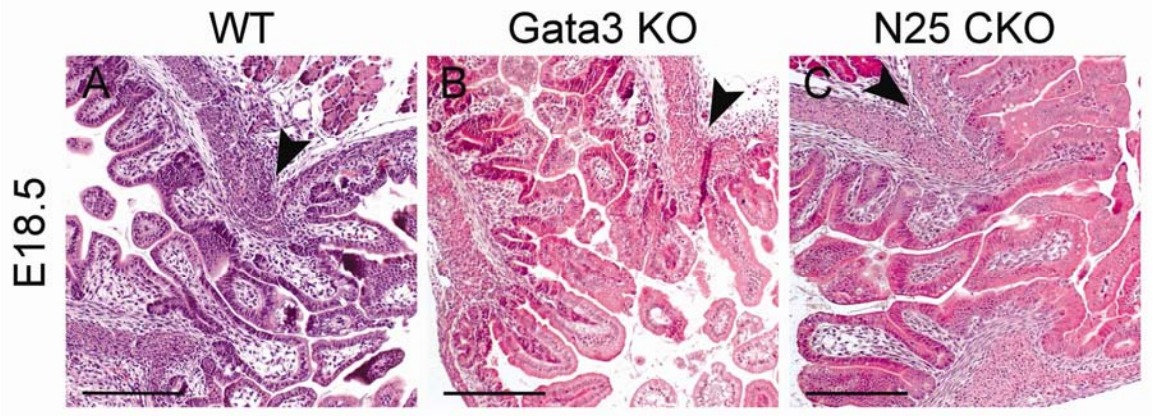

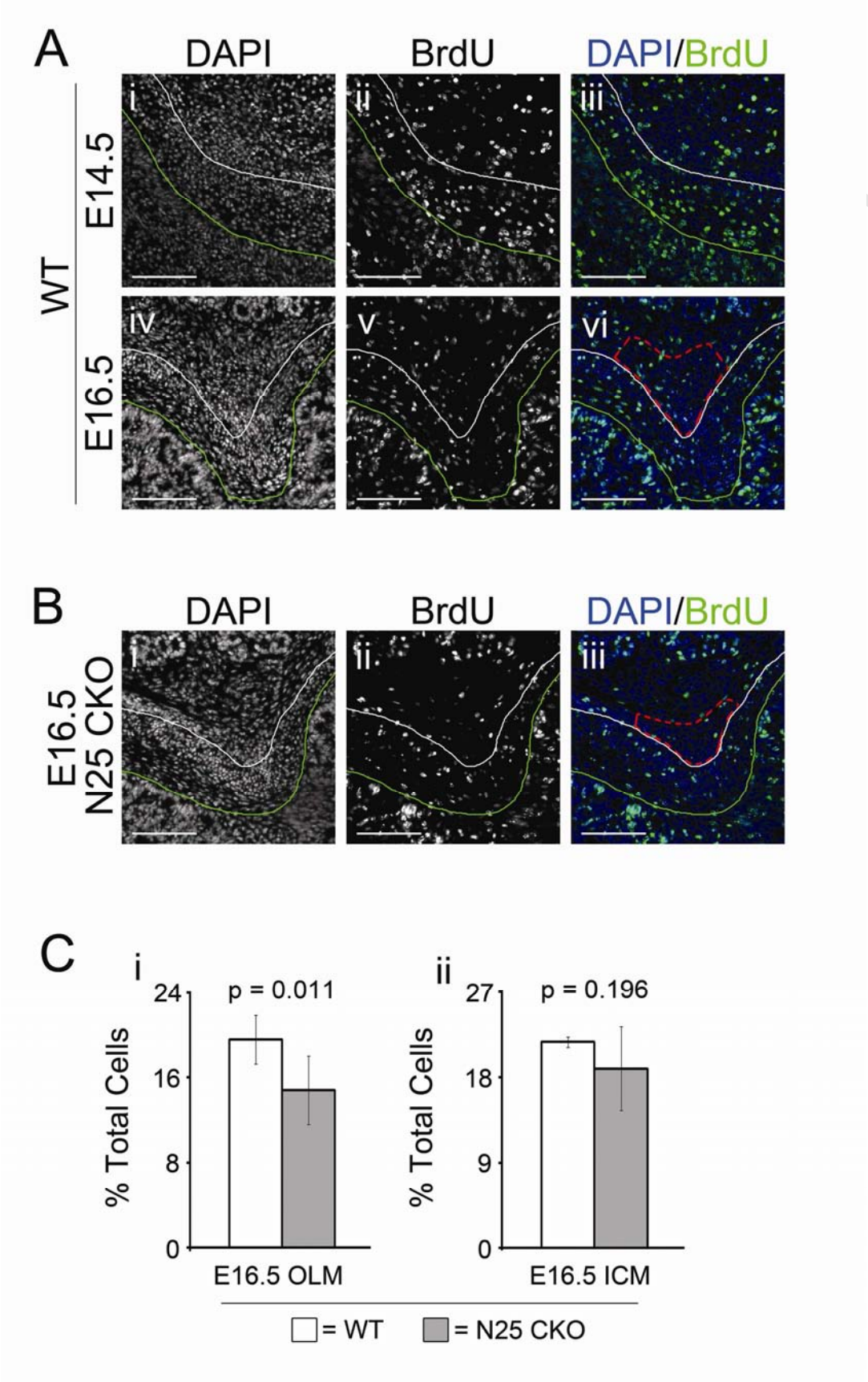
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ii
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E16.5
N25 CKO
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p = 0.011
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E16.5 OLM
ii
p = 0.196
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% Total Cells
E16.5 ICM
= WT
= N25 CKO