Role of the Homeodomain Transcription Factor Bapx1 in Mouse Distal Stomach Development

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**Background & Aims:** Expansion and patterning of the endoderm generate a highly ordered, multiorgan digestive system in vertebrate animals. Among distal foregut derivatives, the gastric corpus, antrum, pylorus, and duodenum are distinct structures with sharp boundaries. Some homeodomain transcription factors expressed in gut mesenchyme convey positional information required for anterior-posterior patterning of the digestive tract. Barx1, in particular, controls stomach differentiation and morphogenesis. The Nirenberg and Kim homeobox gene Bapx1 (Nkx3-2) has an established role in skeletal development, but its function in the mammalian gut is less clear. **Methods:** We generated a Bapx1Cre knock-in allele to fate map Bapx1-expressing cells and evaluate its function in gastrointestinal development. **Results:** Bapx1-expressing cells populate the gut mesenchyme with a rostral boundary in the hindstomach near the junction of the gastric corpus and antrum. Smooth muscle differentiation and distribution of early regional markers are ostensibly normal in Bapx1Cre/Cre gut, but there are distinctive morphologic abnormalities near this rostral Bapx1 domain: the antral segment of the stomach is markedly shortened, and the pyloric constriction is lost. Comparison of expression domains and examination of stomach phenotypes in single and compound Barx1 and Bapx1 mutant mice suggests a hierarchy between these 2 factors; Bapx1 expression is lost in the absence of Barx1. **Conclusions:** This study reveals the nonredundant requirement for Bapx1 in distal stomach development, places it within a Barx1-dependent pathway, and illustrates the pervasive influence of gut mesenchyme homeobox genes on endoderm differentiation and digestive organogenesis.

Mechanisms responsible for organizing the mammalian stomach into fundus, corpus, and antral-pyloric segments are poorly understood. Corpus epithelium typically carries numerous oxyntic and zymogenic cells that produce acid and digestive enzymes, respectively. The distal stomach, which encompasses the antrum and pylorus, lacks these cell lineages but is marked in mouse and man by presence of endocrine cells that secrete gastrin and mucous cells that produce mucin. Muscle cells in the outer pylorus create a sphincter that controls passage of food into the duodenum.

The digestive tract differentiates in response to signals from adjacent mesenchyme. Expression of homeobox genes is often segmental along the anterior-posterior axis of the developing gut and may be especially important in relaying rostro-caudal position. Clustered Hox genes, for example, are expressed in the gut in overlapping domains, reminiscent of patterns observed along the skeletal axis; they are implicated in regional identity and in formation of intestinal sphincters and the cecum. Homeodomain proteins participate in mesoderm-endoderm signaling.

The homeobox gene Barx1 is confined to embryonic stomach mesenchyme and is required for proper stomach development. In its absence, the stomach is markedly small, abnormally shaped, lacks a pyloric constriction, shows mixing of cells from different segments, and carries intestinal villi distally. Some homeobox genes regulate fibroblast growth factor expression in the hindgut, and overexpression of NKK2.5 in chick embryos inhibits Wnt5a and Bmp4 expression during formation of the hindstomach (gizzard) and pylorus. Barx1 acts in part by limiting the duration of Wnt signaling in early stomach development. Many other factors that regulate genetic and tissue interactions in stomach development remain unknown.

The homeodomain of mammalian Nkx3-2 (Bapx1) shares ~87% identity with Drosophila BAGPIPE, a NK2 subfamily member that specifies gut smooth muscle in flies. Viral misexpression studies in the chicken suggest that Bapx1 functions in development of the gizzard, a muscular, keratinized structure in the posterior stomach. In mouse embryos, Bapx1 messenger RNA (mRNA) appears first in lateral plate mesoderm, adjacent to gut endoderm, around embryonic day (E) 8.5.
out mice were therefore predicted to have gut musculature defects, but the intestine in 3 separate mutant lines is largely intact, and investigation has centered on Bapx1’s role in spleen and skeletal development. One group commented on abnormal gastro-duodenal morphology without investigating molecular details. Although the nature and possible reasons for the defect are unknown, Bapx1 is cited as being required to generate pyloric sphincter muscle.

We created a targeted mouse line that marks Bapx1-expressing cells and eliminates gene activity. Here, we report that Bapx1 is necessary for proper antral-pyloric morphogenesis and development of antral-type epithelium. We also show that Bapx1 expression in the distal stomach requires Barx1. These studies reveal a focal requirement for Bapx1 in hindstomach organogenesis and outline a transcriptional hierarchy in mammalian stomach development.

Materials and Methods

Mouse Gene Targeting

A λ-phage clone from a 129/Sv mouse genomic library was provided by Drs. K.-I. Yoshiura and J. Murray, University of Iowa (Iowa City, IA). A 3.6-kilobase (kb) BglII-SaclII fragment containing 5’ flanking sequences and the first 46 codons of Bapx1 exon 1 served as the 5’-homology arm; a 1.6-kb SmaI fragment containing codon 112 through the end of exon 2 served as the 3’-homology arm. A PGK-Neo cassette and Cre recombinase complementary DNA (cDNA) were inserted in frame with Bapx1 coding sequence at the SacII restriction site (Figure 1A). The construct was electroporated into AB2.2 embryonic stem cells. Two targeted cell lines were used to produce chimeras and Bapx1+/Cre mice. For Southern genotyping, the probe was a [α-32P]dCTP-labeled BglII-Sacl fragment from the 3’ segment of the gene, which identifies 5-kb and 7.5-kb bands for wild-type and Cre knock-in alleles, respectively (Figure 1B). To demonstrate correct targeting at the 5’ end, we used primers complementary to the Cre insert (CRE3: GCCGCTAAACCAGTGAACACCAT-TGC) and to genomic DNA ~4.5-kb 5’ to the Bapx1 gene (GTTATGAGTGACGGCTGGGGG) to amplify a 5.7-kb DNA fragment in the targeted allele (Supplementary Figure 1). Identity of this fragment was confirmed by BglII/ClaI digestion and sequence analysis using internal primers GGTTTCAAAATGAGGCTC and CATGTATGAATGTGTGGAAACCTGG and to genomic DNA ~4.5-kb 5’ to the Bapx1 gene (GTTATGAGTGACGGCTGGGGG) to amplify a 5.7-kb DNA fragment in the targeted allele (Supplementary Figure 1). Identity of this fragment was confirmed by BglII/ClaI digestion and sequence analysis using internal primers GGTTTCAAAATGAGGCTC and CATGTATGAATGTGTGGAAACCTGG. For subsequent genotyping, we used CRE3’ along with CTCGT- TCTTCCGCTAGGGCTGAG and CCAGCGATCTCTCAACAAGAGAGGG in a coupled polymerase chain reaction (PCR) reaction with a 56°C annealing step (Figure 1C). Bapx1 targeted mice were maintained on the C57BL/6 background.

Figure 1. Generation of Bapx1Cre targeted mice and their application to examine Bapx1 gene expression. (A) Molecular strategy to generate ES cells with Cre recombinase targeted to the first Bapx1 exon between the SacII and SmaI sites. (B) Mice were genotyped by Southern hybridization using probes corresponding to the 3’ half of exon 2 and part of the 3’ untranslated region. Subsequent generations were genotyped by PCR. (C, D) Confirmation that Bapx1 expression is extinguished in Bapx1Cre/Cre mice. qRT-PCR on fetal and newborn tibial RNA, expressing Bapx1 mRNA content relative to Gapdh mRNA in wild-type (WT, n = 10), Bapx1Cre+ (Het, n = 14), and Bapx1Cre/Cre (KO, n = 10) samples. (E–G) Bapx1Cre+ mice were crossed with the ROSA26R reporter strain, and progeny were examined for Cre-expressing cells and their descendants by β-galactosidase activity. In E12.5 embryos, staining was detected in sites of documented Bapx1 expression or function, including condensing vertebrae (arrowhead) and gut mesenchyme (arrow) (G). Staining also appeared in E13.5 cardiac outflow tract (F) and, later, throughout the developing skeleton (see Supplementary Figure 1). OFT, cardiac outflow tract; RV, right ventricle; LV, left ventricle; CC, central canal; NT, neural tube; N, notochord; S, sclerotome.
Expression Analyses

β-galactosidase activity was determined on whole-mount preparations using published methods. For histology, embryos were embedded in paraffin, sectioned at 5 μm, and stained with H&E. Bapx1Cre/+ and Barx1+/− mice were intercrossed to obtain compound homozygotes. Organs from crosses with Nlxl2.5-GFP transgenic mice, described previously, were visualized under a Leica MZ FLIII fluorescent dissecting microscope.

RNA was reverse transcribed using SuperScript (Invitrogen, Carlsbad, CA). cDNA was detected by PCR using Bapx1 primers aga tgtcagccagcgtttc and gcagagccgagcagctg. Fetal stomach lysates were resolved by SDS-PAGE. Binding of Bapx1 mouse antiseraum H0000579-A01 (1:500; Abnova, Taipei, Taiwan) was detected with horseradish peroxidase-conjugated goat anti-mouse antibody (Ab).

Embryos were incubated in 4% paraformaldehyde at 4°C overnight. Eight-micrometer-thick paraffin sections were dried, deparaffinized in xylene, and rehydrated. For antigen retrieval, slides were immersed in 10 mmol/L sodium citrate, pH 6.0, and treated in a pressure cooker for 2 minutes at 15 psi. Endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 15 minutes and nonspecific Ab binding with 5% fetal bovine serum for 1 hour at 25°C. Primary Ab: SM/Barx1 (Sigma A2547 [1A4], 1:1000; Sigma-Aldrich, St. Louis, MO), PGP9.5 (1:2500; MBL International, Woburn, MA), intrinsic factor (1:24,000; gift from Dr. D. Alpers; Washington University, St. Louis, MO), Pdx1 (1:6000; gift of Dr. C. Wright, Vanderbilt University, Nashville, TN), BMP4 (1:300, Chemicon AB1761), H/K-ATPase (D032-3; 1:1000; Sigma-Aldrich, St. Louis, MO), PGP9.5 (1:2500; MBL International, Woburn, MA), intrinsic factor (1:24,000; gift from Dr. D. Alpers; Washington University, St. Louis, MO), Pdx1 (1:6000; gift of Dr. C. Wright, Vanderbilt University, Nashville, TN), BMP4 (1:300, Chemicon AB1761), and Nkx2.5 (1:200, SC14033; Santa Cruz Biotechnology, Santa Cruz, CA) were applied for 4 hours at 25°C or overnight at 4°C. After treatment with anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) then avidin-biotin complex solution (Vector Laboratories, Burlingame, CA) for 1 hour at 25°C, color reactions were developed in 0.05% 3,3‘-diaminobenzidine and 0.1% H2O2. Slides were counterstained with hematoxylin. Alcian blue staining was by standard methods.

For in situ hybridization, tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich), dehydrated, embedded in paraffin, and sectioned at 6-μm thickness. Deparaffinized, rehydrated sections were treated with Proteinase K (Roche, Pleasanton, CA) then avidin-biotin complex solution (Vector Laboratories, Burlingame, CA) for 1 hour at 25°C or overnight at 4°C. After overnight hybridization at 63°C, slides were washed for 2 hours in decreasing concentrations of SSC from 2X to 0.2X at 63°C then incubated in 5% serum in phosphate-buffered saline followed by digoxigenin Ab (1:2000; Roche) at 4°C overnight. Slides were equilibrated in 100 mmol/L NaCl; 100 mmol/L Tris, pH 9.5; and 50 mmol/L MgCl2 and stained (NBT/BCIP tablets; Roche) for 2–4 hours.

For expression arrays, RNA was extracted from the distal stomach of E18.5 Bapx1Cre/+ embryos and wild-type littermates using the RNeasy kit (Qiagen, Valencia, CA). After confirmation of RNA quality, samples were processed and hybridized to CodeLink mouse bioarrays (Amersham Biosciences, Piscataway, NJ). Raw data were normalized on a log scale and filtered to reduce noise. Differential gene expression and functional gene groupings were analyzed using MatchMiner (http://discover.nci.nih.gov/matchminer/), GoMiner (http://discover.nci.nih.gov/gominer/), and GeneSpring (Agilent Technologies, Santa Clara, CA) software and are deposited in the GEO database (GSE 13935).

Results

Tracing Bapx1 Expression

We used homologous recombination to replace Bapx1 at codon 46 (exon 1) with in-frame Cre cDNA (Bapx1Cre, Figure 1A). Two independent mutant lines showed evidence for correct gene targeting (Figure 1B and 1C) and no material effect on expression of the 2 flanking genes (Supplementary Figure 1C). We crossed these mice with ROSA26 reporter mice, in which a floxed translation-stop sequence restricts LacZ gene expression to Cre-expressing cells and their progeny. β-galactosidase (β-gal/LacZ) activity first appeared in Bapx1Cre;ROSA26R embryos at E9.5 in gut mesoderm and weakly in somites (Figure 2A and 2B; data not shown). Between E10.5 and E12.5, β-gal activity was prominent in the splanchnic mesoderm, somites, calvarium, Meckel’s cartilage, and spleen anlage (Figures 1E, 2C and 1D, and Supplementary Figure 2A–2G). By E13.5, Bapx1 expression was evident in the cardiac outflow tract (Figure 1F) and condensing cartilage of the ribs, skull (Supplementary Figure 2I–2N), and long bones. Staining in the digestive tract was confined to mesodermal derivatives and excluded from endoderm at all stages (Figures 1G and 2E and 2F). These findings agree with previous reports of Bapx1’s role in developing skeleton and spleen17–20 and establish the fidelity of Bapx1Cre mice to mark Bapx1-expressing cells and elucidate Bapx1 function in other organs.

Definition of gene expression along the long axis of the embryonic stomach is confounded by rotation of the organ from an initial lie parallel to the body’s anterior-posterior axis to a final position that is nearly perpendicular. We examined serial embryo sections with the attention required to distinguish the stomach’s antero-posterior and radial axes. LacZ expression in Bapx1Cre/+ embryonic gut initiated in the distal stomach. Staining at E10.5 was intense in the caudal foregut and stomach-intestine junction but absent
from the rostral foregut and stomach (Figure 2E, sections from the same embryo in a rostral to caudal series). At E11.5, expression remained evident in the hindstomach but faint or absent in forestomach (Figure 2F), extended into the full-length of intestine, and included the spleen anlage (Figure 2F). LacZ staining involved all cells in the full thickness of the mesenchyme (Supplementary Figure 3). In older embryos, β-gal activity was present in much of the stomach, with a persistent caudal-to-rostral gradient (data not shown).

Early chick embryos express Bapx1 in the prospective gizzard (posterior stomach) but not the proventriculus (anterior stomach). Our Cre-based lineage analysis in mice confirmed Bapx1 expression in tissues with known functions and disclosed an anterior boundary previously unappreciated in mammalian stomach. The rostral limit of earliest Bapx1 expression corresponds roughly to the junction between corpus and antrum.

**Abnormal Stomach Development in Bapx1Cre Homozygotes**

Crosses between Bapx1+/Cre mice yielded null mutants in Mendelian proportions until E18.5 (25.6% Bapx1+/+, 48% Bapx1Cre/+, and 26.4% Bapx1Cre/Cre). Mutant homozygotes typically died 1 to 3 days after birth, and ~90% of weanlings were wild-type or heterozygote. Perinatal lethality, similar to that reported with other Bapx1-null alleles, likely reflects skeletal malformation (Supplementary Figure 2O and 2P). The spleen was absent or markedly hypoplastic in Bapx1Cre/Cre mice, as judged grossly (Figure 3B) and by expression of a Nkx2.5-
Expression and distribution of PGP9.5, an enteric ner-

tologic sections (C and D) of E16.5 and neonatal specimens, respec-
tively. Marked truncation of the antral-pyloric segment of the stomach
(brackets) and lack of the pyloric constriction (arrowheads) are readily
evident. The spleen (Sp) is also absent or markedly hypoplastic in
Bapx1Cre/Cre mice. (E–H) Immunohistochemical analysis of PGP9.5
(E and F) and smooth muscle α-actin (G and H) in Bapx1Cre/Cre neonatal
hindstomach indicates ostensibly intact enteric nerve and smooth muscle
differentiation, respectively. Arrows point to immunostaining of gan-
glia (E and F) or smooth muscle (G and H). Red bars demarcate the
pylorus and highlight the marked difference in width between control and mutant samples.

GFP transgene (Supplementary Figure 4), a marker of the
developing spleen.12,23

Bapx1Cre/Cre stomachs were modestly reduced in size. Nearly all of this reduction occurred in the distal seg-
ment, which was also dilated and lacked constriction at the gastro-duodenal junction, the site of the pyloric
sphincter (Figure 3A and 3B). Histologic examination confirmed distal dilatation and revealed severe shorten-
ing of the antral-pyloric segment (Figure 3C and 3D). Expression and distribution of PGP9.5, an enteric ner-

vous system marker,26,27 and smooth-muscle α-actin were intact (Figure 3E–3H).

Epithelia in the gastric body and antrum have distinct-
tive features. Specialized, Alcian blue-avid mucous cells
found at the base of antral gland units are normally absent from the corpus; conversely, the antrum lacks
chief and oxyntic cells, the dominant lineages in the body.1 Normal hindstomach hence corresponds to the
Alcian blue-staining region between the zone of chief and parietal cells in the corpus and the villous duodenal
epithelium (Supplementary Figure 5A). Bapx1Cre/Cre stom-
achs carried few, and in many cases, no glandular units
with basal Alcian blue avidity (Figure 4A and 4E vs 4B
and 4F), whereas intestinal goblet cells stained readily
with Alcian blue (Figure 4F). Additionally, the distance
between H/K-ATPase– (Figure 4C and 4G vs 4D and 4H)
or gastric intrinsic factor (Supplementary Figure 5B–E)
expressing cells in the stomach body and the villous
intestinal epithelium was markedly reduced. These

Correlates of Bapx1 in

Indian Hedgehog (Ihh) mRNA is enriched in fetal
mouse corpus and antrum, whereas Sonic hedgehog (Shh) is
enriched in the forestomach.28 In a sign that early pat-
terning is preserved in Bapx1Cre/Cre stomach, the bound-
aries of Ihh (Figure 5A and 5B) and Shh (data not shown)
expression were intact at E11.5. Expression of the ho-
meobox gene Pdx1 is normally limited to the antral-
pyloric segment, providing a reliable marker of this stom-
ach region.29 Pdx1 expression was also similar in Bapx1Cre/Cre
and wild-type stomach early in development (E11.5 and
E14.5 shown in 5E–5H), indicating diminu-
tion or loss of mature antral character. Scrutiny of Alcian
blue, H/K-ATPase, and gastric intrinsic factor stains re-
vealed normal cell composition in distal corpus glands
and absence of mixed corpus-antral units (Supplemen-
tary Figure 5F).

Thus, absence of Bapx1 leads to significant hindstom-
ach truncation and loss of the pyloric constricti

Molecular Correlates of Bapx1 in

Hindstomach Development

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and wild-type stomach early in development (E11.5 and
E14.5 shown in 5C–5F), Consistent with the observation
of antral hypoplasia, the Pdx1 expression domain was
substantially smaller at E18.5 (data not shown). However,
the typical transition in staining pattern between antrum and corpus, and the symmetry across greater and lesser curvatures, were preserved. Antral hypoplasia in the absence of Bapx1 hence occurs on the background of correct anterior-posterior stomach patterning.

In chick embryos, Nkx2.5 and Bapx1 are expressed in the distal stomach (gizzard), whereas Bmp4 and Wnt5a appear in the proximal proventriculus and are excluded from the gizzard.\(^\text{15,30}\) Nkx2.5 may regulate pyloric sphincter development, and forced Bapx1 expression in the proventriculus inhibits endogenous Bmp4 expression.\(^\text{15}\) In mouse embryos, by contrast, we observed Bmp4 expression throughout stomach and intestinal mesenchyme (data not shown); Nkx2.5 mRNA and protein were also expressed widely in mesoderm at the gastroduodenal junction but clearly enriched in pyloric sphincter muscle, as predicted (Figure 5G and Supplementary Figure 2). Levels and distribution of both Nkx2.5 (Figure 5H, Supplementary Figure 4) and Bmp4 (data not shown) were unaltered in Bapx1\(^{Cre/Cre}\) stomach, indicating that Bapx1 loss does not interfere with their expression.

Next, we surveyed changes in Bapx1\(^{Cre/Cre}\) antral gene expression, using microarray analysis followed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) confirmation of representative results (data not shown). Because early stomach pattern seems intact, we reasoned that antra from older embryos would better reveal aberrant gene expression. The distal stomach in


**Table 1B**). These molecular changes in distal \( Bapx1^{Cre/Cre} \) stomach represent an unknown combination of additional regional markers and possible underpinnings of antral hypoplasia.

**Bapx1 May Function Downstream of Barx1 to Mediate Antral-Pyloric Development**

Bapx1 is coexpressed in embryonic hindstomach mesenchyme with Barx1, although the domain of Barx1 expression encompasses nearly the whole stomach (Figure 6A). Both genes influence differentiation of overlying stomach endoderm and formation of the pyloric sphincter; the antral segment is abbreviated in \( Bapx1^{Cre/Cre} \) mice (Figures 3 and 4) and likely lost in \( Barx1^{-/-} \) mice.\(^{12}\) We crossed mice to produce compound homozygote mutants, which we studied immediately after birth because \( Barx1^{-/-} \) mice die of respiratory failure in the perinatal period.\(^{12}\) Stomach anomalies in \( Barx1^{-/-};Bapx1^{Cre/Cre} \) and \( Barx1^{-/-};Bapx1^{+/-} \) neonates were identical (Figure 6B and 6C); there was no worsening of the isolated \( Barx1 \) mutant phenotype, which is more severe than the \( Bapx1^{Cre/Cre} \) antral defect.

To evaluate further the relationship between these coexpressed factors, we investigated gene expression in each individual knockout strain. Levels and distribution of \( Barx1 \) mRNA were not reduced or altered in fetal \( Bapx1^{Cre/Cre} \) stomachs and may even increase slightly (Figures 6D and 6E, Supplementary Table 1A). Thus, Barx1 does not require \( Bapx1 \) for its expression and acts either upstream or independent of \( Bapx1 \). Conversely, we detected \( Bapx1 \) transcripts in wild-type hindstomach and spleen (Figure 6F, arrow and arrowhead) but not in the caudal \( Barx1^{-/-} \) stomach (Figure 6G, arrow), indicating that hindstomach \( Bapx1 \) expression requires \( Barx1 \) function. \( Bapx1 \) expression was equally robust in wild-type and \( Barx1^{-/-} \) somites (Figure 6G, inset), ruling out trivial reasons for lack of a stomach signal. qRT-PCR and immunoblot analyses confirmed that \( Bapx1 \) mRNA levels were markedly reduced or absent in \( Barx1^{-/-} \) stomachs (Figure 6H and 6I). These observations collectively suggest that \( Bapx1 \) expression depends on \( Barx1 \) and that antral dysmorphogenesis in \( Barx1^{-/-} \) stomachs might potentially reflect the attendant \( Bapx1 \) deficiency.

**Discussion**

Organogenesis requires positional cues to specify cell and tissue types correctly. Homeobox genes play a vital role in regulating developmental processes and imparting positional identity.\(^{31,32}\) We used homologous recombination to drive Cre expression from the mouse \( Bapx1 \) locus, thus creating a new null allele to define expression and study gene function in the developing gut. \( Bapx1^{+/-};ROS26R \) mice confirmed \( Bapx1 \) expression domains reported previously in cartilage and spleen and revealed that, early in digestive tract development, \( Bapx1 \)-expressing cells and their progeny are confined to

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Figure 5. Molecular correlates of embryonic stomach pattern and pyloric development imply normal patterning early in \( Bapx1^{Cre/Cre} \) stomach development. (A–F) Markers of hindstomach endoderm, \( Ihh \) mRNA (A and B), and Pdx1 protein (C–F), showed no difference in expression between wild-type and \( Bapx1^{Cre/Cre} \) stomachs at E11.5 (A–D) or E14.5 (E and F). Dashed arrows mark the rostral limit of \( Ihh \) expression, which is much stronger in hindstomach (HS) and corpus (Cp) compared with forestomach (FS); solid arrows mark the anterior boundary of PDX1 expression, at the corpus-antral junction. PDX1 is also expressed in proximal intestine (Int) and pancreas (Pa), as seen especially clearly in the control samples (C and E). (G and H) Expression of the chick hindstomach and pyloric determinant \( Nkx2.5 \) mRNA in \( Bapx1^{Cre/Cre} \) and wild-type stomachs at E11.5. \( Nkx2.5 \) expression, which marks developing pyloric sphincter muscle (arrows, dashed lines), was unaffected by \( Bapx1 \) loss. Similar results were observed by immunostaining (E14.5, data not shown). ISH, in situ hybridization; IHC, immunohistochemistry.

\( Bapx1^{Cre/Cre} \) embryos at E18.5 showed an increase in corpus-specific \( H/K-ATPase \) and \( Gif \) transcripts and a corresponding decrease in antrum-specific \( Muc6 \) mRNA (Supplementary Table 1A). These changes are consistent with the loss of antral, and distal extension of corpus, character. Considering functional gene classes (Gene Ontology), we noted increased expression of transcripts in groups related to epithelial-mesenchymal transition and regulation of endocytosis, whereas groups associated with Smad proteins, nuclear protein import, and vesicle membranes were expressed at lower levels (Supplementary
the intestine and prospective hindstomach. In line with this observation, Bapx1Cre/Cre mice show significant shortening of the antral segment and virtual apposition of the gastric body to the duodenum. Pdx1 and Ihh, 2 posterior markers, show correct regional expression, implying that certain elements of early stomach patterning are preserved. Thus, Bapx1Cre/Cre hindstomach defects seem to reflect a failure of proper expansion and morphogenesis.
of the antral-pyloric segment. Because the affected region corresponds to that where Bapx1 expression initiates in the digestive tract, we infer that Bapx1 activity is uniquely responsible for these aspects, even if the precise molecular mechanism is presently unknown.

Despite ostensibly normal smooth muscle differentiation and preserved expression of Nkx2.5, a gene implicated in chick gizzard development,30 Bapx1Cre/Cre mice also lack normal pylorus morphology. Mice deleted for nearly the full Hoxd gene cluster lose multiple gastrointestinal valves, including the pyloric sphincter, with associated changes in regional smooth muscle and mucosa; the pyloric constriction is also missing in Barx1−/− mice.12 These findings may be relevant to hypertrophic pyloric stenosis, a common congenital disorder.33 Future efforts should aim to understand how these homeobox genes interact to generate the pyloric sphincter.

The stomach corpus and intestine developed normally, indicating that the antrum-pylorus is the only gut segment that requires Bapx1 for proper development. Alternatively, Bapx1 may function redundantly with other homeobox genes elsewhere. Less likely, abnormal hindstomach development could reflect dysmorphogenesis of the spleen and pancreas. Around E8.5 in mouse development, Bapx1 mediates lateral growth of the splanchic mesodermal plate and coupled leftward growth of the dorsal pancreas, associated with control of Fgf10 expression.34 However, anomalies akin to those we identify in Bapx1Cre/Cre stomach are not seen with a wide range of defects in spleen and pancreas development.12,25 In chondrocytes, Bapx1 serves both proliferative and antiapoptotic roles,19,35 and one reason the antrum and pylorus may develop abnormally in its absence is if hindstomach progenitors are disadvantaged relative to anterior cells programmed for corpus differentiation. Immunostaining for cleaved caspase 3 did not reveal excess apoptosis in E11.5 hindstomachs (data not shown).

Absence of Barx1 markedly disrupts stomach development, producing aberrant morphogenesis, intestinal homeosis, and pyloric sphincter agenesis. Additional loss of Bapx1 does not worsen this phenotype, and the greater severity of antral-pyloric defects in the Barx1 mutant hints at actions upstream of Bapx1. Indeed, Bapx1 expression is virtually lost in Barx1-null stomach, and its absence could potentially account for some part of the Barx1−/− phenotype in the distal organ. Mice with tissue-specific loss of a third stomach transcription factor, the nuclear hormone receptor COUP-TFII, also show a mild patterning defect.38 Besides expansion and disorganization of circular smooth muscle and enteric neurons, the margin between forestomach and corpus is shifted anteriorly, and the glandular stomach accordingly occupies a larger relative space. Although expansion of the corpus is a common feature of the 2 phenotypes, they occur at opposite ends: anteriorly in the case of COUP-TFII deficiency and posteriorly in Bapx1Cre/Cre animals.

Bapx1 is expressed throughout stomach mesenchyme, whereas Bapx1 is initially confined to the caudal region. Thus, although Barx1 seems to be required for stomach Bapx1 expression, it cannot be sufficient to restrict expression to the hindstomach; other factors may promote Bapx1 expression caudally or repress it rostrally. We are presently investigating Barx1’s role in COUP-TFII expression. Our results meanwhile implicate Barx1 and Bapx1 within an essential pathway for mammalian hindstomach development.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.01.009.

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Conflicts of interest
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