# Development and Pathology of the Mammalian Pylorus

By

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# Introduction

## Summary

The focus of this thesis is the pylorus, an anatomical sphincter that controls the passage of food from the stomach to the duodenum. Malformation or malfunction of this sphincter results in two significant pathologies: idiopathic hypertrophic pyloric stenosis (IHPS) occurs when the pylorus is obstructed or tonically closed, while duodenogastric reflux (DGR) is a consequence of failure to properly close the pyloric sphincter. Neither the developmental history of the pylorus nor the molecular pathoetiologies that underlie these severe pathologies are clearly understood; the work described here was undertaken further explore both of these aspects. In this first chapter, I will summarize what is known about the anatomy of the pyloric region, its embryonic development and the genetic network involved in its maturation. Additionally, I will describe the major pyloric pathologies in terms of their presentation, epidemiology, diagnosis and treatment. Since at least in the case of IHPS, a strong genetic component has been clearly demonstrated, I will also review some of the genetic associations that have been implicated in that condition. This will set the stage for the presentation of my experimental work in Chapters 2 and 3. In Chapter 4, I will consider the implications of the data that I have gathered in the context of pyloric development and disease, and suggest future studies that could impact the diagnosis and treatment of IHPS and DGR and shed additional light on the factors involved in pyloric ontogeny.

## 1.1 Major divisions of the gastrointestinal tract

The gastrointestinal tract of the mouse and human are structurally very similar [1]. Food enters through an oral cavity and is propelled down the short, straight esophagus by the action of swallowing (Figure 1.1A). A gastroesophageal sphincter controls the entry of food into the stomach, where it is partially digested by the actions of gastric acid and proteases. The pyloric sphincter, which is positioned between the stomach and small intestine, regulates progression of the partially digested food, known as chyme, into the small intestine, where further digestion and absorption take place. Several specialized anatomical terms are used to describe specific areas of the stomach and intestine and are important in the context of this thesis. In the mouse, the most anterior portion of the stomach, called the forestomach, is histologically an extension of the esophagus; it is covered by squamous, non-keratinized epithelium an epithelium able to withstand the coarse diet of the mouse, which may contain hair and dirt. Humans lack this specialized forestomach region. The glandular stomach (also called “body”, “corpus” or “fundus”) is the most anterior region in the human, but located in the central area of the stomach in the mouse. In the chick, another model in which pyloric development has been well-studied, this glandular region is called the proventriculus. In all species, the glandular stomach is the site of secretion of acid as well as digestive enzymes like pepsin.

The posterior stomach, referred to in humans and mice as the antrum (or pyloric stomach), is an important site of mucus production (Figure 1.1B); this heavy mucus layer protects the pylorus and nearby duodenum from the acid produced by the corpus. In the chick, this region is called the gizzard. It is highly muscular and may contain stones and grit; its function is to grind the food, serving part of the function of the teeth, which are lacking in the chicken. The muscular

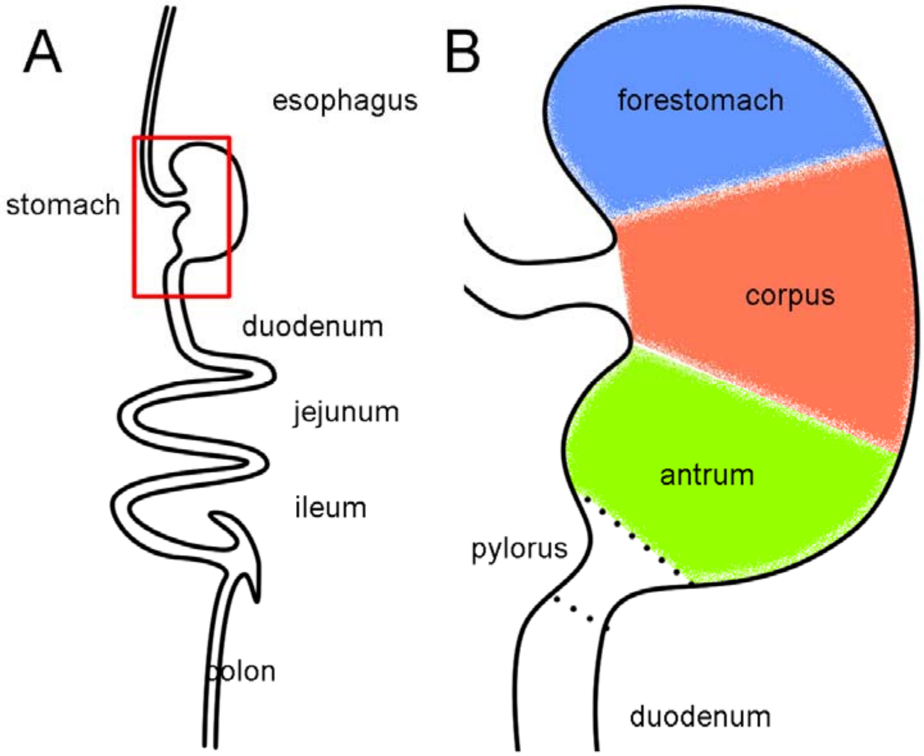

*Figure1.1 The normal mouse gastrointestinal system*
*(*A) Food travels from the oral cavity, through the esophagus, and undergoes mechanical disruption and acid breakdown in the stomach. Then it is further digested and absorbed while traveling through the three regions of the small intestine: the duodenum, jejunum, and ileum. Finally, the food is dehydrated in the colon, where colonic bacteria break down important nutrients and prepare stool for excretion. (B) An inset of the stomach and proximal duodenum (red box, A). The stomach is divided into three portions, the anterior forestomach, the corpus or fundus, and the distal antrum. The antrum is connected to the proximal duodenum via the pylorus.

pyloric sphincter controls gastric emptying to the small intestine. Posterior to the pylorus, the small intestine is divided into distinct regions from anterior to posterior: these are termed the duodenum, jejunum, and ileum.

The focus of this thesis is the pylorus. Functionally, the pylorus not only regulates food passage between the antrum and duodenum by allowing the regular emptying of the stomach, but additionally, since it is tonically constricted except when signaled to open, it serves to prevent the backflow of duodenal contents into the stomach [2]. The pylorus is a true, or anatomic, sphincter; that is, it is associated with a muscular thickening that can adjust the lumenal diameter using muscular tone. This is in contrast to the so-called "physiological" sphincter that defines the transition from the esophagus to stomach, which controls flux using a pressure gradient generated by the diaphragm. At the pyloric sphincter, constriction is regulated by a complex and coordinated series of inputs from the nervous system, hormonal signaling, interstitial cells of Cajal (pacemaker cells) and intrinsic muscular tone. Abnormalities in the sphincter or in any of these inputs could potentially result in pyloric dysfunction.

Two clinically significant diseases of pyloric function have been described: infantile hypertrophic pyloric stenosis (IHPS) and duodenogastric reflux (DGR). The first is thought to arise from tonic closure of the pylorus, preventing the passage of food to the duodenum, while the latter is a consequence of inability to maintain pyloric closure, thus allowing backward movement of duodenal contents and bile into the stomach. While most individuals probably suffer periodically from DGR, congenital DGR is a rare condition, for which there is no known molecular underpinning [3, 4]. In contrast, IHPS is one of the most common birth anomalies and its genetic basis is just beginning to emerge [5-8].

### 1.2 Pyloric morphology

Like the rest of the GI tract, the pylorus is composed of cells from all three germ layers. The epithelium is derived from endoderm, while the underlying lamina propria and muscular components are mesenchymal. Neuronal cells of ectodermal origin innervate the pylorus and control its function. In the avian model, the epithelium in the region of the pylorus is distinct from that of the nearby gizzard and duodenum [5]. Gizzard epithelial cells have a keratin layer and long cilia, likely to resist the abrasive grinding critical in the normal function of the avian gizzard. In contrast, the avian pyloric epithelium lacks the keratin layer and has short, bulbous cilia, while the duodenal epithelium lacks cilia entirely. Interestingly, the adult mammalian pylorus lacks a unique pyloric epithelium. Rather, the pylorus is the site of an abrupt junction between antral and duodenal epithelial cell types [6]. As measured by intestinal markers such as *Cdx2* and villin, this epithelial boundary is one cell thick: at the pylorus, a cell expressing multiple intestinal markers lies immediately adjacent to cells expressing stomach markers [6]. This distinct boundary evolves from an earlier, more graded expression pattern in which cells expressing gastric markers (SOX2) overlap for a short distance at the pylorus with cells that express intestinal markers (e.g., *Cdx2*) [7]. The conversion of this graded border to the sharp boundary of epithelial cell types characteristic of the adult occurs at E16.5 in the mouse [6].

The pyloric mesenchyme is composed of several concentric layers. The lamina propria is the layer closest to the epithelium; it contains blood vessels, immune cells, lymphatics, fibroblasts and myofibroblasts. A thin muscularis mucosa underlies the epithelium and lamina propria. The submucosal layer is not well developed at the pylorus; rather it is dominated by the muscularis externa, consisting of an inner circular muscle (ICM) and an outer longitudinal muscle (OLM). The myenteric plexuses of the enteric nervous system lie in between the ICM and OLM (Figure 1.2).

The inner circular muscle of the GI tract forms a circumferential sheath around the tubular gut, with the fibers of the muscle oriented perpendicularly from the anterior-posterior (A-P) axis. The ICM forms at approximately embryonic day (E) 11, as judged by increasing expression of the muscular protein alpha smooth muscle actin [8]. The OLM arises 24 to 48 hours later, with fibers oriented down the length of the gut tube. The thickness of these muscular layers is variable throughout the length of the GI tract. The gastric ICM is relatively thick and arranged into fascicular bundles. It thickens considerably at the pylorus with the fascicles oriented obliquely relative to those of the stomach. The ICM thins considerably on the duodenal side of the pylorus and changes relatively little throughout the rest of the small intestine. The OLM undergoes similar morphological changes but with a vastly decreased thickness relative to the ICM.

A complex network of innervations control the contraction or relaxation of the pyloric sphincter through excitatory and inhibitory signals, respectively [9, 10]. The extrinsic innervations at the pylorus include both sympathetic and parasympathetic neurons from the peripheral nervous system. Sympathetic stimulation at the pylorus is primarily excitatory, while parasympathetic innervations can have either an excitatory effect through cholinergic signaling or an inhibitory effect through vasoactive intestinal peptide (VIP) and nitric oxide (NO) signaling. The majority

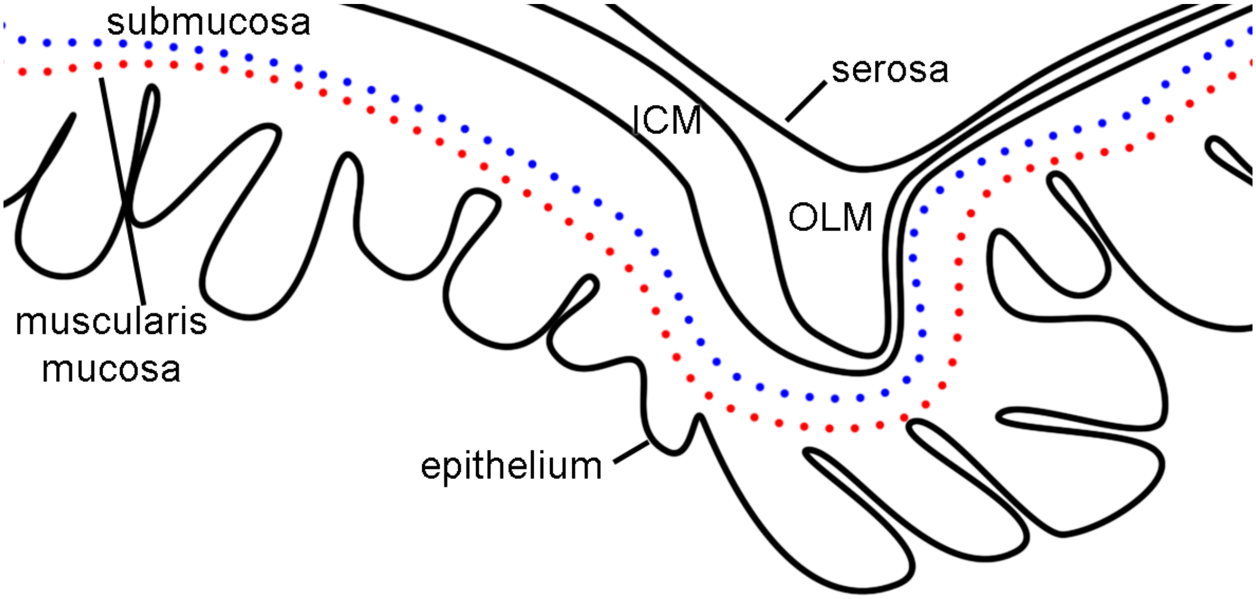

*Figure 1.2 Schematic of the embryonic pylorus*
A schematic of the E16.5 embryonic pylorus illustrates its component layers. The epithelium transitions from the glandular epithelium of the antrum (left) to the villus epithelium of the duodenum (right). The region known as the mucosa includes epithelium, lamina propria and underlying thin muscularis mucosa (red dotted line). Below the mucosa lies the submucosa, filled with connective tissue (blue dotted line) and the two layers of the muscularis externa, the ICM and OLM. Between these muscular layers are the neurons of Auerbach's myenteric plexus. Squamous serosal (mesothelial) cells cover the surface of the stomach, pylorus and intestine.

of the fibers at the pylorus appear to be fibers of the vagus nerve carrying afferent sensory signals [13]. These fibers relay signals to the CNS from the muscle stretch receptors. Interestingly, studies show that the net extrinsic innervation at the pylorus is inhibitory, with denervation of the pylorus resulting in decreased flux of solid food content [2]. In addition, the myenteric plexus within the muscularis externa provides the intrinsic innervation to the pylorus [11]. The ganglia of the myenteric plexus are large and embedded between the two layers of the muscularis mucosa. They provide either excitatory signals through enkephalins and substance P or inhibitory signals through VIP and NO [9]. Interstitial cells of Cajal serve as the pacemaker cells which lead to the basal contractile rhythm of the GI tract. At the pylorus, these cells appear to form gap junctions with each other and with smooth muscle rather than with nervous extensions [10], suggesting an important role for cell-cell contacts in maintaining pyloric constriction.

### 1.3 Pyloric Pathologies

Having summarized normal pyloric physiology, I will describe the two primary pathologies of the pylorus: duodenogastric reflux and infantile hypertrophic pyloric stenosis. These pathologies have been well described functionally in both the clinic and in animal models, with surgical interventions for IHPS being described as early as the turn of the 20th century [12]. However, there is still a significant amount of ongoing investigation into the pathogenesis of both diseases. New molecular markers and genetic assays are providing insights into the varied etiologies by which DGR and IHPS arise [13]. Here I will describe the clinical presentation of both illnesses and then describe the current understanding of these etiologies.

### 1.3.1 Duodenogastric Reflux

Duodenogastric reflux results from a defect in pyloric closure and is therefore the phenotypic or functional opposite of IHPS.  Mild duodenal reflux into the stomach likely occurs in nearly everyone at some time and is normally asymptomatic. Pathologic DGR occurs when this reflux is excessive or occurs extensively over a period of time [3]. There are a number of overlapping definitions of the nature of the reflux, including reflux of duodenal contents into either the stomach or duodenum, and reflux of biliary contents anywhere along the biliary tree. Here, I will focus on describing the clinical presentation DGR, where duodenal contents reflux into the stomach, causing inflammatory changes in the gastric mucosa, called bile reflux gastritis (BRG). I will then explore the pathogenesis of this disease in an animal model.

#### *Clinical presentation of DGR*

Early studies of DGR primarily identified this pathology as resulting from a number of surgical interventions, including gastric surgery, pyloroplasty, and cholecystectomy [4, 14]. The condition was often defined by clinical presentation, with bilious vomit being the first clue that a chronic reflux problem was occurring. Analysis of the gastric contents in fasting patients frequently demonstrated bile acid and the gastric mucosa was likely to show several prototypical changes: foveolar hyperplasia, vascular congestion, edema, smooth muscle infiltration, and a lack of inflammatory cells. This presentation is distinct from the chronic gastritis found in patients colonized with *Helicobacter pylori* infection. In fact, several studies have demonstrated that bile reflux actually decreases the prevelance of *H. pylori* and partially resolves the chronic inflammatory response [15-18]. Subsequent studies in normal patient populations showed that there exists a significant population of patients that present with primary BRG (BRG without prior midgut surgery) [19, 20]. These patients present with similar symptoms as the post-operative patients, including nausea, vomiting, and epigastric pain. Pediatric patients differ from their adult cohort only in that there is a significant negative correlation between vascular congestion and DGR in children, in direct contrast to the earlier findings. These studies leave several unanswered questions regarding the presentation and progression of this disease. In addition, there is no clear correlation between the degree of DRG and the nature of the histological changes in the stomach [15, 21].

*Pathogenesis of DGR*

The causes underlying the development of primary DGR are currently unclear and the existing studies are fairly limited. The only mouse model is the $W/W^v$ mouse, which exhibits loss of c-kit and therefore loss of the interstitial cells of Cajal, the pacemaker cells of the gut [22]. Extensive bile acid staining of the gastric mucosa in these mice, when studied post-mortem, was taken as diagnostic of DGR. An orthologous rat model, the $W^s/W^s$ rat, shows similar reflux symptoms, with increased concentrations of bile acid in the stomach [23]. While the specific mechanism by which increased bile acid causes metaplastic gastritis is still unclear, several studies have shown correlative links between the two pathologies [3, 17, 21]. Increased bile acid reflux is associated with marked intestinal metaplasia in the stomach. Specifically, the formation of absorptive, goblet, or Paneth cells within the gastric mucosa. This is generally believed to be the first step in the progression toward gastric cancer and greatly increases the likelihood of developing gastric cancer [15, 17].

### 1.3.2 Infantile Hypertrophic Pyloric Stenosis

IHPS is a developmental disorder of the pylorus. Several excellent reviews on IHPS have recently been published [24]; the most salient aspects from those reviews are highlighted here. IHPS is relatively common, reported to occur in 1.5 to 3 in every 1000 live births, with slight variations depending on social and ethnic demographics [9, 24-26]. Interestingly, IHPS is much more common in males, with a male:female ratio of 4-5:1, though the reason for this is unclear. Clinical symptoms of IHPS typically arise 3-5 weeks after birth, with rare cases arising as soon as the first few weeks of life. Infants typically present with spitting, which can progress to projectile vomiting following feedings, sometimes so forceful that vomitus is ejected from the nose as well. It may contain brownish, "coffee ground" material, likely the dried remnants of GI bleeding. The infant may also present with wasting and dehydration, but this is dependent on the severity and duration of the symptoms. The tonically closed pylorus can be palpated; a pyloric mass or "olive," is present upon physical examination in 70-90% of affected infants [27]. Diagnosis is confirmed by abdominal ultrasound, in which the pylorus appears as a bright donut. IHPS can be successfully treated surgically. A procedure called a pyloromyotomy is done to relieve the tension on the pylorus (Figure 1.3; [28]). Specifically, a longitudinal incision is made along the greater curvature of the hypertrophic pylorus, with subsequent blunt dissection to the level of the submucosa. Complete healing takes place within approximately a month and this seems to restore proper function to the pylorus. Complications from this procedure are rare but can include duodenal perforation, infection, gastroesophageal reflux, and vomiting [29, 30].

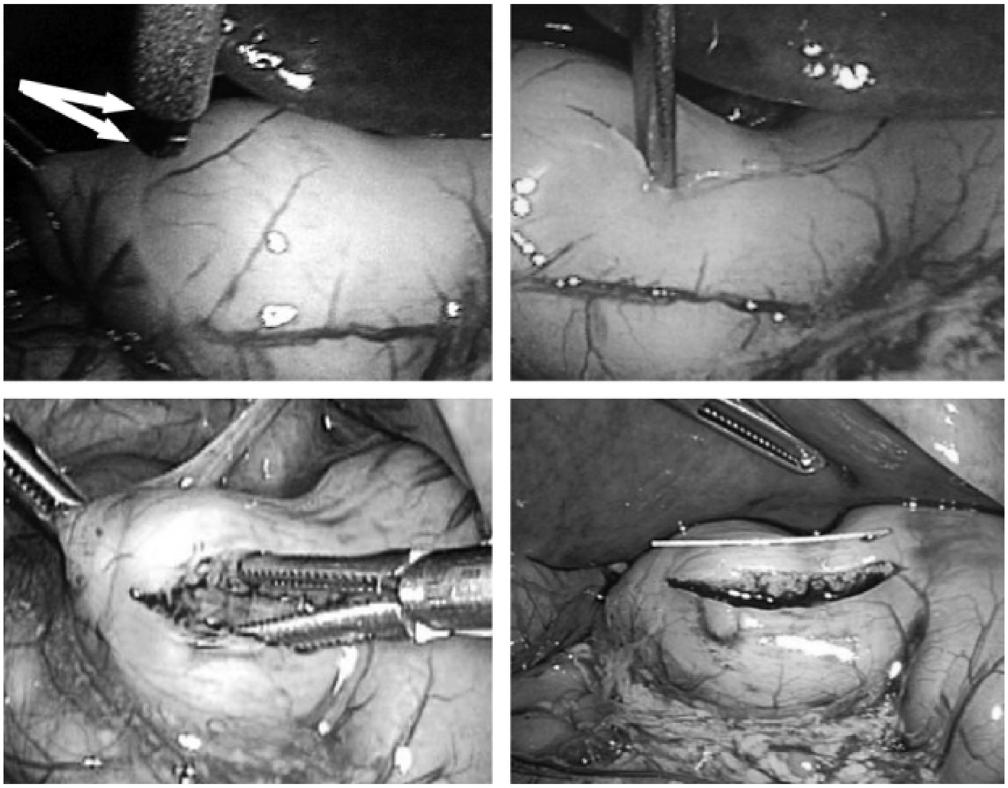

*Figure 1.3 Laparoscopic pyloromyotomy*
*Images taken from a laparoscopic pyloromyotomy demonstrate the longitudinal incision made along the hypertrophied pyloric musculature with the retractable pyloromyotomy blade (white arrows). Then the pyloric spreader is used to complete the surgery. The lower right image shows the completed procedure and extent of the incision (white string = 2 cm). (Image reprinted from St. Peter et al. 2006 with permission from Wolters Kluwer Health).*

Much is known about the epidemiology and presentation of IHPS and, properly surgically treated, it seldom results in fatality. However, significant morbidity is associated with this condition, justifying a continued search for its cause. Muscular, nervous, hormonal and epithelial pathologies have all been postulated in IHPS, but the various studies have found disparate and conflicting evidence, possibly suggesting that several unique subtypes of IHPS exist.

### *Muscular pathology*

Obvious attention has been paid to possible abnormalities in the muscles that form the pyloric sphincter. In some cases of IHPS, swollen smooth muscle cells with dilated rough endoplasmic reticulum, excess glycogen, and abnormal, enlarged mitochondria have been documented [32, 33], suggesting that pyloric obstruction could result from cellular hypertrophy. However, a subsequent study found roughly normal smooth muscle cells, with only an increase in rough endoplasmic reticulum at the pylorus [31]. This latter study also reports that the smooth muscle cells in IHPS are more commonly in a proliferative state than those in the normal pylorus, implicating hyperplasia instead of hypertrophy as a mechanism behind pyloric stenosis. In support of these findings, another study found that IHPS cells express increased amounts of the protein desmin, a protein that is highly expressed during myogenesis [32]; the authors speculated that the increased desmin expression might be a sign of a poorly differentiated (fetal-like) muscle. This study was contradicted a few years later, by a group that showed that the muscular proteins desmin and alpha-smooth muscle actin were expressed at similar levels in control and IHPS patients. Rather, the cytoskeletal proteins talin and dystrophin were absent in IHPS [33]. Reduced levels of these proteins could lead to impaired cell-matrix and membrane-cytoskeletal interactions, respectively, resulting in loss of pyloric myogenic structural integrity and tone. In support of altered interactions between muscle cells and the extracellular matrix, a more recent study demonstrated severely reduced sarcoglycan expression [34]. Sarcoglycan is an important component of the dystrophin/glycoprotein complex that acts to link the muscle cell to the extracellular matrix. Taken together, these data make clear that there is no current consensus on a single mechanism by which alterations in the muscular tissue lead to IHPS, and it is possible that several myogenically-based etiologies could give rise to this condition.

### *Neuronal pathology*

Like the abnormalities seen in the smooth muscle cells, many studies of neuronal abnormalities in IHPS have focused on the histopathologic findings. Changes in the shape, number, and size of the ganglion cells of the myenteric plexus have been documented in IHPS patients by some authors [35-38], while normal ganglion number and morphology were seen by others [39-41]. Changes in nerve cell bodies and axons have also been seen [39, 42]. Finally, alterations have been observed in the contacts between nerve and muscle within the pylorus. Reduced numbers of neuromuscular contacts were observed in one study [40] and another reported reduced expression of the adhesion molecule NCAM within the pyloric muscle itself, but not in the myenteric plexus . Thus, it is possible that alterations in the innervation proper or in the synapses between nerve and muscle contribute to IHPS.

As mentioned earlier, several different types of neurotransmitters are involved in pyloric signaling, and several of these have been implicated in the pathogenesis of IHPS. One study shows that acetylcholinesterase expression decreases in the muscular layers in IHPS tissue relative to controls [43]. This increases cholinergic activity at the pylorus and, consequently, pyloric excitation. Other studies have shown a marked decrease in adrenergic staining IHPS patient tissue, suggesting that the relative expression of these transmitters could be significant in determining the course of this disease. Additionally, decreases in both the excitatory neurotransmitters enkaphalin and substance P as well as the inhibitory neuropeptides vasoactive intestinal polypeptide and neuropeptide Y have been observed [44, 45]. Panteli speculates that the loss of inhibitory neurotransmitters could underlie the inability of the pyloric muscle to relax, and the resulting hyper-activated pyloric tissue could consume excitatory neuropeptides [9].

Perhaps the most compelling case for a causative role of a neurotransmitter-like molecule in IHPS is that of nitric oxide (NO), an important cell signaling molecule that can also regulate muscular tone. NO is generated from L-arginine by neural nitric oxide synthase (nNOS); it is believed to act as an inhibitory factor that can stimulate smooth muscle relaxation [46, 47]. A number of authors have documented sustained and marked decrease in nNOS in IHPS pyloric tissue [22, 48]. One study that followed patients post surgically for the treatment of IHPS found that systemic nitrite levels were significantly decreased in IHPS patients before pyloromyotomy, but normal afterwards, suggesting that the surgical treatment not only relieves symptomatic pyloric pressure but alters signaling modalities at the pylorus [48]. Interestingly, a mouse model of nNOS deficiency has been described; these mice exhibit pyloric hypertrophy [22]. The same phenotype was seen after administration of a NOS inhibitor to pregnant rats, suggesting that loss of NO alone may be enough to cause IHPS [49]. Together, these data indicate that NO signaling is critical in pyloric relaxation and its absence may induce pyloric hypertrophy.

Another possible etiology for IHPS is loss or malfunction of the interstitial cells of Cajal (ICC). These cells function as electrical pacemakers, networking with neuronal and muscle cells to control coordinated peristaltic contractions along the gut tube. Both electron microcopy and immunostaining for the ICC marker, *c-kit*, indicate that the ICC network is disturbed or reduced in IHPS [42, 50, 51]. Interestingly, ICC might be required for both nitrergic and choninergic responses, since both responses were reduced in c-kit mutant $W/W^v$ mice that lack ICC [52]. Thus, NO signaling might require ICC and given that NO donors seem to improve ICC survival [53], this dependent relationship may be critical to the pathoetiology of IHPS. However, as is the pattern with studies on IHPS etiology, a more recent study partially contradicts these findings, finding no evidence that ICC are involved in nitrergic inhibition [22]. In that study, pyloric sphincter function was measured manometrically and a zone of high pressure was detected at the sphincter that was reduced in $W/W^v$ mice but normal in iNOS null mice. In the latter animals, the loss of NO resulted in lack of inhibition by vagal stimulation; indeed, a paradoxical excitation was detected, which could explain the delayed gastric emptying and beazors seen in this model. In contrast, $W/W^v$ mice showed normal nitrergic inhibition in this study and exhibited bile in the stomach, characteristic of duodenogastric reflux.

### *Hormonal alterations*

Though studies in the 1970's suggested that administration of gastrin to dogs could produce a condition like pyloric stenosis [54], a connection between gastrin and IHPS has not been reproducibly demonstrated in humans. Other hormones that could influence the function of the pyloric muscle include somatostatin, secretin, cholecystokinin, prostaglandins and vasoactive intestinal peptide (VIP). All of these have been investigated in various studies. Though two case reports suggest that treatment with prostaglandins can induce pyloric stenosis [55, 56], this has not been systematically investigated and the existing evidence for a causal link between any of the other hormones and IHPS is not compelling (reviewed in Panteli).

### *Mucosal etiology*

With much attention paid to other possible etiologies, discussed above, few studies have carefully examined the mucosal aspect in cases of IHPS. One extensive study looked at 102 cases of IHPS and found a consistent thickening of the mucosa, with a mean mucosal thickness measured on sonographs of 4.1 ± 0.9 mm in IHPS patients as compared to 2.3 ± 0.5 mm in 51 controls [57]. Biopsy of one patient showed an abnormally folded epithelium with edematous submucosa and inflammation. This histological picture was similar to that shown after prostaglandin-induced stenosis, described above [56], a finding that is interesting in light of the fact that increased prostaglandin levels have been detected in IHPS [58].

### *Pathoetiology of IHPS: in search of the cause*

Taken together, all of these data make clear that there is no current consensus on a single mechanism by which IHPS arises, and it is possible that several etiologies could give rise to this condition. It is also important to keep in mind that most of these studies examine the disease endpoint in patients, well after the inciting factor has acted. Because it is very difficult, if not impossible, to deduce cause from effect in this manner, there is a great need to generate animal models in which IHPS can be reproducibly generated and studied.

## 1.4 Genetics of IHPS

The epidemiology of IHPS makes it likely that this condition arises from genetic susceptibility, exacerbated by some environmental/physiological stimulus (e.g., hormonal changes that accompany birth, feeding, increased gastric acidity, etc.). Family studies have revealed a strong genetic component, with a 200 fold increase in IHPS incidence in monozygotic twins and a 20-fold increase in siblings or dizygotic twins [59]. A more recent genetic analysis of IHPS used a genome-wide association study (GWAS) to identify genomic loci associated with inherited IHPS [13]. By analyzing the presence of single nucleotide polymorphisms (SNPs) in 1001 individuals with IHPS and 2401 controls, the authors found three specific SNPs that were the most significantly associated with IHPS. These SNPs were located 150kbp upstream of the gene *MBNL1*, near the genes *C3ORF79* and *ARHGEF26*, and between the genes *BNIP1* and *NKX2-5*. Several other SNPs were also noted to be associated with an increased prevalence of IHPS, including the SNP rs2228671 on chromosome 19p13.2, which is correlated with the significant excess risk of IHPS in males [12, 59, 60]. However, this association could not be replicated and so was not further pursued. Another recent study confirmed two of the novel loci for IHPS on chromosomes 3 and 5 [61]. These data suggest that the genes *MBNL1* and *NKX2-5* are the most interesting targets for further study.

### *MBNL1 is associated with postnatal splicing*

*MBNL1* is one of a family of three muscleblind proteins (MBNL1-3) that were originally described in association with myotonic dystrophy (DM) [62, 63]. These proteins are homologues of the *Drosophila* muscleblind protein, which is required for terminal differentiation of muscle tissue and photoreceptors [64, 65]. Analysis of the function of these genes revealed that they are involved in mutant RNA accumulation in the nucleus, suggesting a role in transcription [66].

Subsequent studies demonstrated that the MBNL genes are responsible for regulation of alternative splicing of cardiac troponin T and insulin receptor in DM [67]. These genes are also antagonized by the CELF proteins, which are also implicated in DM pathogenesis. Significantly, both of these gene families have been implicated in alternative splicing regulation during postnatal cardiac development [68, 69]. During the course of cardiac development, *MBNL1* expression rises at around postnatal day 5 (PN5) [68]. This correlates with the expression of a subset of genes that show splicing defects in DM and are expressed between P2 and P20 [69]. As I mentioned earlier, IHPS incidence occurs 2-8 weeks after birth in humans. This corresponds closely to the timepoint at which *MBNL1* is found to be critical for the regulation of DM gene expression. Thus, it may be that a mutation in *MBNL1* that alters the expression of a downstream splicing target at around this time gives rise to IHPS.

### *NKX2-5 is a marker of splenic precursor cells*

In the GWAS study, participants with a SNP found near the *NKX2-5* locus were 42% more likely to have IHPS than controls [13]. *Nkx2-5* is a homeodomain transcription factor expressed in several organ domains including the heart, pylorus, thyroid, and pharynx [70-72]. It is a known regulator of cardiomyocyte differentiation [73-75], and a marker of splenic precursor cells [76, 77]. I will discuss its known expression in the early lateral plate mesoderm and spleen morphogenesis in section 4.2. Expression of *Nkx2-5* at the pylorus is highly conserved in chick, mouse and human [78-80], and has been implicated as a regulator of epithelial morphogenesis at the chick pylorus (Figure 1.4, see Section 5.1). The known functions and expression domains of *Nkx2-5* make it a promising gene for further investigation in mammalian pylorogenesis.

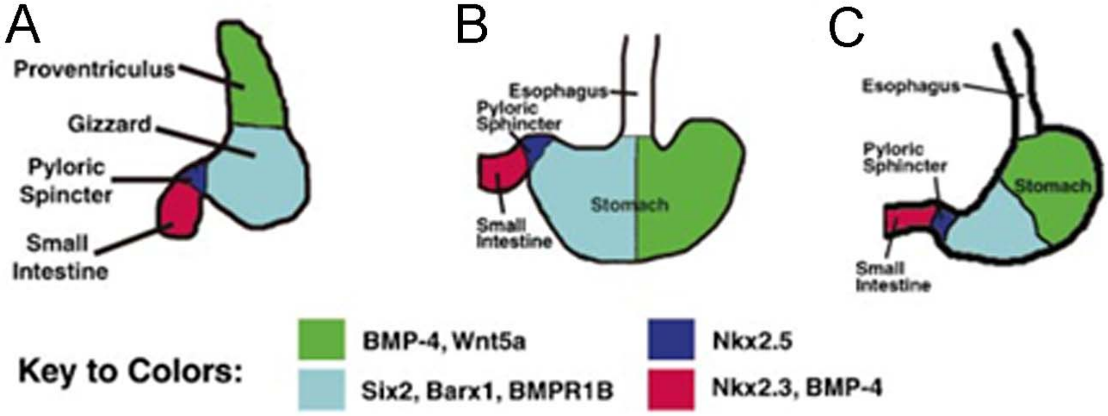

*Figure 1.4 Patterning of the forgeut-midgut junction is evolutionarily conserved* Schematics representing signaling molecule and transcription factor expression domains in the (A) chick, (B) mouse, and (C) human stomachs show broad conservation of patterning across species. In all of these model systems, *Six2* and *Barx1* are expressed in an antral domain while NKX2-5 is expressed at the pylorus. The signaling molecule *Bmp4* is expressed in both the stomach and duodenum but is excluded from the pylorus. (Image modified from Smith et al. 2008 with permission from John Wiley & Sons).

*Other implicated genes*

The other genes near the implicated loci within the GWAS study have a variety of functions not closely associated with muscle development. C3ORF79 is a transcript of unknown function thus, while it may be related to IHPS, this relationship must be explored further. ARHGEF26 encodes the gene SGEF, a guanine nucleotide exchange factor that has been implicated in atherosclerosis as factor that recruits and retains leukocytes at athero-prone sites of inflammation [81]. In addition, it has been associated with the promotion of prostate cancer development and progression [82]. Given the existing data on IHPS pathogenesis, it may be that an inflammatory pathway contributes to the disease. But further investigation is required to determine whether or not *SGEF* plays a role in IHPS.

A locus on chromosome 19 segregates with IHPS, particularly with males [59]. The closest gene to this locus is a member of the YIP1 family, *YIPF2*. While there are no published data on this gene, other YIP1 family members have been described as critical regulators of vesicle biogenesis, that mediate association with the well known vesicle trafficking Rab proteins [83, 84]. Loss of YIP1p leads to severe disruption of membrane transport and ER vesicle budding [85]. Since studies of IHPS have described alterations in endoplasmic reticulum formation and function [31, 42], it is possible that this gene may be involved in at least some etiologies of IHPS. The chromosomes on which the most highly associated loci were found were chromosomes 3 and 5. A microarray analysis of foregut-midgut junction genes performed by our lab identified several proteins that were expressed in a pyloric-specific domain [7]. Of these, the gene *NR2C2* is expressed on human chromosome 3, about 150kb upstream of a strongly associated SNP [13] (Supplemental). This gene codes for the protein testicular receptor 4 (*TR4*), an orphan nuclear receptor that is involved in a number of signaling pathways including the retinoic acid (RA) signaling pathway [81, 82], and the PPARα signaling pathway [83, 84]. Due to the heterogeneity of the upstream modulators and targets of this gene, it is difficult to speculate regarding its putative role in the pathogenesis of IHPS.

### 1.5 Embryonic and fetal development of the pyloric region

While linkage studies are providing some interesting candidate genes that might functionally contribute to the development of IHPS (and DGR), testing the function of those genes in mouse models is required in order to confirm their involvement and understand their roles. Such analyses in turn require a solid understanding of the development and patterning of pyloric tissue components. Like the rest of the GI tract, the epithelium at the pylorus is derived from endoderm while the underlying stroma and muscle is mesodermally derived. The composition of adult pyloric tissue is described above; in this section, I review the early embryonic and fetal events that lead to development of the pyloric muscle and its overlying epithelium.

Patterning of the mammalian digestive system begins with regional expression of the Nodal transcription factor in a region of the early embryo called the proximal epiblast. During gastrulation, an endodermal fate is promoted in cells with high Nodal and mesodermal fate in cells with low Nodal [85]. Around E8.5, the endoderm transforms from a flat sheet to a cylindrical gut tube. This occurs through the formation of the anterior intestinal portal (AIP) and the caudal intestinal portal (CIP) which then migrate caudally and rostrally, respectively. By the end of this process, when the gut endoderm is a simple cylindrical tube, it is already patterned into broad anterior, medial, and posterior domains identified by the expression of a variety of

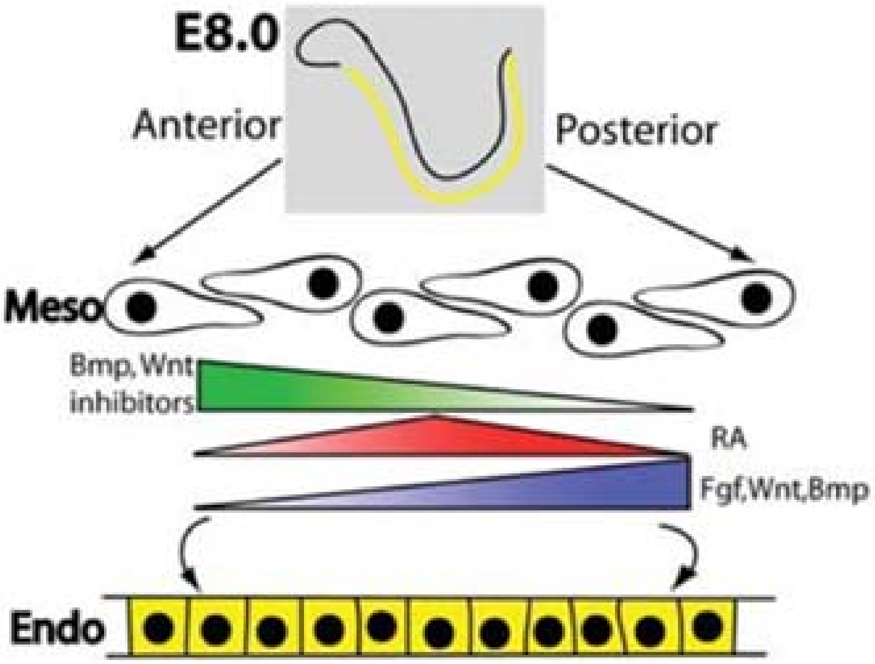

*Figure 1.5 Signaling pathways in the developing mouse endoderm*
Schematic of the BMP, WNT, FGF and RA pathways in the developing endoderm shows that the BMP, WNT, and FGF pathways are expressed in an increasing anterior-posterior gradient. Conversely, inhibitors of the BMP and WNT pathways are expressed in a decreasing anterior-posterior gradient. In contrast, the RA pathway is expressed in an increasing gradient that peaks at the midgut and decreases again toward the posterior hindgut. (Image reprinted from Spence et al. 2011 with permission from John Wiley & Sons).

transcription factors including *Sox2*, *Pdx1*, and *Cdx2*. These transcription factors are critical in the establishment of the foregut-midgut boundary.

A variety of signaling pathways are involved in endodermal organogenesis, including Hedgehog, Bmp, Wnt, Fgf, and RA signaling pathways (Figure 1.5; [85-91]). The expression of each of these transcription factors is domain specific and they work in concert to establish the precise A-P pattern of the gut and its associated organs. During foregut specification, high Fgf induces *Nkx2-1* expression in the trachea and lung progenitors, moderate Fgf induces Albumin in liver progenitors, while low Fgf induces *Pdx1* expression in pancreatic progenitors [91]. This gradient is similar in direction and function to that of the signaling molecule Bmp. During this time, RA signaling is also necessary for the establishment of foregut fates. *Raldh2* null mice fail to develop foregut organs including the lungs, stomach, and dorsal pancreas [92]. RA appears to function both independently through Hox genes and through downstream effectors including the Fgf and Hh pathways [86].

### 1.5.1 Foregut/Midgut Organ domains in the epithelium

The broad foregut-midgut boundaries formed during early embryogenesis are necessary for the organ-specific patterns that arise in mid to late development. In the epithelium, SOX2 expression is induced throughout the foregut region and has been shown to be necessary for the formation of both the esophagus and lungs. Specifically, severe *Sox2* hypomorphs develop a variety of tracheoesophageal fistulas with proximal esophageal atresia [93]. Similarly, expression of *Cdx1* and *Cdx2* are important for the specification of intestinal epithelium. Loss of *Cdx2* leads to anteriorization of the intestinal epithelium, with a stronger phenotype with compound deletion of both *Cdx1* and *2* [94]. This process leads to the expression of anterior endodermal markers in the intestine, including the genes *Sox2* and *Barx1*, the latter of which is involved in defining the gastric phenotype [95], and will be discussed in detail in section 5.2.

The pylorus actually forms at the boundary between the stomach and duodenum (marked early on by the boundary of the *Sox2* and *Cdx2* domains in the epithelium). Interestingly, *Pdx1*, a paired family homeobox protein, is expressed in the epithelium of the distal stomach (antrum), pylorus and proximal duodenum. *Pdx1* is known to be important for pancreas development and its loss leads to failure of the pancreatic anlag to develop [96]. Additionally, loss of *Pdx1* causes dysmorphology of the pylorus [97], though the pathological picture has not been well described either histologically or molecularly.

### 1.5.2 Foregut/Midgut Organ domains in the mesenchyme

The formation of the mesenchymal investment at the pylorus is an interesting and dynamic process. Specification of the pyloric mesenchyme begins with the creation of the left-right (LR) asymmetry in the lateral plate mesoderm (LPM) that is initiated by Nodal signaling [76, 98, 99]. The signaling molecules that regulate this asymmetry, including Shh, Fgf-8, and Bmp, are well described [98, 99], but we are only recently beginning to understand the transcription factors that transduce this signal to the tissues themselves.

*Nkx2-5* is expressed in the LPM, initially bilaterally, and later predominantly on the left in the area of the dorsal pancreas outgrowth. This expression domain also marks the site of splenic and pyloric precursor cells [76, 100]. *Nkx2-5* is a homeodomain protein that is known to be involved in cardiomyocyte differentiation; loss of *Nkx2-5* leads to embryonic lethality at E11.5 due to a loss of cardiac looping [101]. *Nkx2-5* expression in the LPM is obvious by E9.5 in two distinct domains that flank the dorsal pancreatic bud [76, 77]. This expression overlaps that of another known splenic gene, *Hox11*, demonstrating that this is truly splenic precursor tissue [76]. Since this LPM tissue gives rise to both the pancreas and the spleen, it is termed the splenopancreatic mesenchyme. The region abutting the splenopancreatic mesenchyme on the left side of the mouse is termed the splanchnic mesodermal plate (SPM).

By E10, *Nkx2-5* expression has solidified into a single, continuous domain with the right and left sides connected dorsally [77]. The presumptive pyloric sphincter forms in this area and, by E10.5, *Nkx2-5* expression is obvious in the pyloric mesenchyme. By E12.5, the *Nkx2-5* positive cells in the region of the pylorus give rise to a stream of cells that then form an elongated structure along the A-P axis of the stomach: the future spleen. The cellular stream between the pylorus and the spleen continues to express *Nkx2-5* until around E14.5, when the spleen and pylorus form two distinct regions of *Nkx2-5* expression [77]. The further expression and function of *Nkx2-5* at the pylorus will be discussed in section 5.1 and 5.2.

### 1.5.3 *Bapx1* is necessary for proper formation of the splenopancreatic mesenchyme

At E10.5, the homeodomain protein *Nkx3-2* (*Bapx1*) is also expressed in an asymmetric pattern in the SPM [106], along with the gastric gene *Barx1* [76]. *Bapx1* null embryos are asplenic, suggesting that this early expression is critical for spleen formation. Interestingly, loss of *Bapx1* also results in the dorsal pancreatic bud growing at the midline, instead of laterally as it normally does, suggesting that loss of *Bapx1* leads to dysfunction of the entire splenopancreatic mesenchyme. Indeed asplenic mouse models generally show a marked disruption of the gastric lobe of the pancreas [104, 107].

## 1.6 Pyloric gene expression

As described above, development of the pyloric domain is a complex process involving overlapping expression domains of a variety of signaling and transcription factors. Once the pylorus is morphologically distinct, a number of genes are expressed in a pylorus-specific pattern, including *Bmp4*, *Nkx2-5*, *Sox9*, *Barx1*, and *Six2*. Studies from our lab have identified a variety of other genes that are expressed uniquely at or around the pylorus [7], but the ones listed above are the best characterized of that set. Here, I will describe their expression and summarize their known functions at the pylorus.

### 1.6.1 Gene expression in the chick pylorus

Our current understanding of pyloric signaling is largely based on early work in the chick embryo. In the chick, the pylorus is the junction between the gizzard (homolog of the antrum) and the duodenum. Placement, and perhaps formation of the pyloric muscle in the chick appears to be regulated by expression of the signaling factor Sonic Hedgehog (*Shh*) [79], which activates expression of its target, *Bmp4* [78, 79, 102]. *Bmp4* is a member of the TGF-β family of signaling molecules which are critical for differentiation and patterning in a variety of organ systems. *Bmp4* expression is apparent in the gut tube as soon as E2.5 in the chick embryo (about E8 in the mouse embryo). Interestingly, *Bmp4* expression appears to be excluded from the region that will form the pyloric sphincter [102]. However, several BMP receptors are expressed at the pylorus throughout this time, with *Bmpr1b* specifically expressed in the developing gizzard mesoderm

[78, 79, 102]. Earlier studies demonstrated that ectopic expression of *Bmp4* in the gastric region leads to decreased mesenchymal muscular thickness [103]. Similarly, viral misexpression of *Bmp4* at the pylorus interferes with muscle development, decreases mseodermal proliferation and increases apoptosis. In addition, ectopic expression of *Bmp4* at the pylorus leads to expansion of the expression domains of both *Nkx2-5* and *Sox9*, suggesting that these genes are BMP targets at the pylorus [78].

NKX2-5 is expressed in a narrow band at the pylorus beginning at least as early as E12.5 [78-80]. Pyloric expression of *Nkx2-5* appears to be regulated by *Bmp4* and in the chick, the function of *Nkx2-5* seems to be specification of the morphology of the overlying pyloric epithelium [78]. When *Nkx2-5* is misexpressed in the chick gizzard, the overlying gizzard epithelium changes character, and becomes similar to pyloric epithelium [78]. Loss of *Nkx2-5* does not alter pyloric epithelial morphology, but causes secretion of koilen, a keratin-like substance that is characteristic of the gizzard, into the pyloric lumen; thus, its loss partially transforms the pyloric epithelium to gizzard epithelium [5, 78]. Neither overexpression or loss of *Nkx2-5* in the chick model has any apparent effect on the mesenchymal tissues, including the muscle.

Like *Nkx2-5*, *Sox9* appears to control epithelial character. In the absence of pyloric *Sox9*, the pyloric epithelium adopts a gizzard-like phenotype and ectopic expression of *Sox9* in the gizzard transforms epithelium to a pylorus-like phenotype [5, 78]. Gremlin1 has been identified as a downstream target of *Sox9*; loss of *Grem1*, like loss of *Sox9*, alters the pyloric epithelium, making it gizzard-like [5]. While both *Nkx2-5* and *Sox9* are known *Bmp4* targets at the chick pylorus, they do not appear to regulate each other in that model. Importantly, there is no evidence in the chick system to connect either *Sox9* or *Nkx2-5* to pyloric muscular development or function.

### 1.6.2 Gene expression in the mouse pylorus

More recent studies have expanded on earlier descriptions of signaling molecules and transcription factors at the mouse pylorus. In particular, a series of studies have implicated the known posterior stomach markers *Barx1*, *Nkx2-5* and *Six2* in pyloric muscular development. In addition, subsequent studies have shown that genes involved in spleen development, like *Bapx1*, are also involved in pyloric patterning. Here I will summarize the known findings on mouse mammalian pyloric development and tie these findings into a larger signaling framework (Figure 1.6).

#### *Barx1 regulates antral patterning*

Earlier I discussed the role of *Barx1* in the establishment of gastric mucosa in heterotopic lesions in *Cdx2* chimera [104]. *Barx1* is a foregut mesenchymal homeobox transcription factor that is expressed throughout development. In *Barx1* null mice, the stomach is shrunken and malformed, with defects in epithelial differentiation and morphogenesis [95, 105]. By E12.5, there appears to be heaped, undifferentiated epithelium and *Cdx2* expression is obvious in the antral epithelium, where it is normally not seen. Loss of *Barx1* also decreases expression of the WNT inhibitors secreted frizzled related proteins (sFRP) 1 and 2, suggesting that WNT inhibition is important in normal stomach development. Indeed, *Axin2* expression is obvious throughout the developing stomach in *Barx1* null embryos. At the pylorus, the constriction that is normally present between stomach and duodenum and marks the position of the pyloric sphincter, is missing. Interestingly,

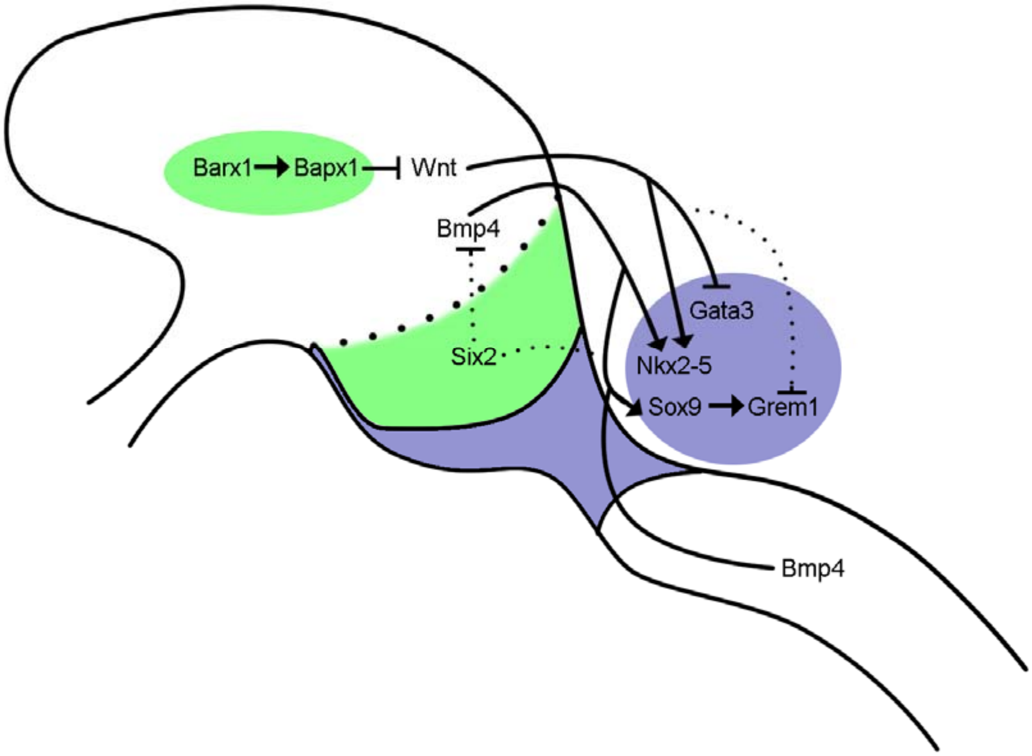

*Figure 1.6 Pyloric Signaling network*
This schematic of the pyloric signaling network shows two overlapping expression domains: the pyloric domain (purple) and the antral domain (green). The former is the site of mesenchymal expression of *Nkx2-5*, *Sox9* and *Grem1*, which have all been shown to be downstream of *Bmp4* in the chick model. The antral domain is the site of expression of *Six2*, *Barx1*, and *Bapx1*, Signaling relationships are summarized from [5, 78, 80, 105, 108].

though the spleen does not express *Barx1*, this organ is mislocalized and hyperplastic in the Barx null animals. This may well reflect the dependence of the spleen on proper pyloric patterning.

### *Bapx1 is downstream of Barx1 in pyloric muscular patterning*

*Bapx1* is a homeodomain protein that was earlier discussed in relation to the formation of the LPM [76]. It is also known to regulate gizzard mesenchymal patterning and development [106]. In the mouse, *Bapx1* (also known as Nkx3-2) is expressed in the mesenteries, midgut mesenchyme and splenic primordium [107] during development. This expression refines to the muscular layers of the GI tract and the mesenteries in the adult mouse. *Bapx1* null embryos lack spleens, and show malformations of the pyloric sphincter. There is a significant loss of constriction with some shape change and loss of thickness in the overlying muscularis mesenchyme [107, 108]. The epithelium and neuronal layers are unchanged in the absence of *Bapx1*, suggesting a specific role in muscular development.

*Bapx1* appears to be downstream of *Barx1*. Both *Barx1* and *Bapx1* are coexpressed in the antral mesenchyme with *Barx1* expression extending throughout the stomach. When *Barx1* is deleted within the *Bapx1* domain, the phenotype is identical to that seen in the absence of *Bapx1*. Interestingly, loss of *Bapx1* does not appear to change the expression of the midgut transcription factors *Nkx2-5* (in pyloric mesenchyme) or Pdx1 (in epithelium).

### *Six2 is necessary for pyloric constriction*

The transcription factor *Six2* also has a conserved antral expression across several different species [1, 80]. *Six2* null embryos exhibit a distinct loss of pyloric muscle and absence of pyloric constriction throughout development. Though both the OLM and ICM are present in *Six2* null embryos, neither muscle layer displays its characteristic pyloric expansion by E18.5. *Six2* also appears to affect BMP signaling as well as *Nkx2-5* and *Sox9* expression at the pylorus. In the absence of *Six2*, *Bmp4* expression expands into the pyloric region, from which it is typically absent. In contrast, *Nkx2-5* and *Sox9* expression appear to be significantly reduced and absent, respectively, in *Six2* null mice, though the reduction in *Nkx2-5* expression seems to be temporary [80]. Loss of *Six2* also leads to a decrease in pyloric Gremlin expression, which is expected if *Grem1* is a target of *Sox9* [5]. Given that these findings contrast with those in the chick pylorus regarding the relationship between *Bmp4*, *Nkx2-5*, *Sox9* and *Grem1*, this would either suggest that the relationship between those genes is different in the mammalian pylorus, or that *Six2* is involved in contrasting ways in different parts of that signaling cascade. Thus, the position of *Six2* in the pyloric signaling cascade is currently unclear.

### *Canonical WNT signaling induces loss of pyloric constriction*

Studies on the function of *Barx1* and *Bapx1* demonstrated that WNT signaling is intrinsically linked to gastric patterning [95, 105]. However, those studies did not definitively demonstrate that inhibition of WNT signaling is directly involved in that patterning process. A subsequent study demonstrated that induction of WNT signaling does indeed lead to alterations in pyloric mesenchymal development reminiscent of those seen in the absence of *Bapx1* [109]. Specifically, ectopic expression of WNT9b or a stabilized β-catenin protein in the antral *Six2* expression domain leads to a loss of pyloric constriction and decreased muscular thickness. In addition, ectopic WNT signaling increases NKX2-5 expression while decreasing the expression of *Grem1* and the transcription factor *Gata3*. That NKX2-5 and *Grem1* expression respond oppositely contrasts with earlier findings in the chick pylorus and in the *Six2* knockout mouse, where these genes are modulated in concert [78, 80]. These findings also contrast sharply with those seen in the *Barx1* and *Bapx1* null models, where NKX2-5 expression appears unaffected even after perturbations to the WNT pathway [105, 108]. The underlying basis for the different results in these two models is unclear.

*GATA3 is a novel pyloric transcription factor*

Work from our lab was the first to identify the expression of the Gata family transcription factor *Gata3* at the pylorus [7]. Before this study, *Gata3* had been described in a number of different tissues throughout the body, including skin, blood, eye, CNS, mammary glands, and adrenal glands [110-117]. While its specific function within these tissues varies, it is broadly required for differentiation and tissue specification in all of these systems. At the pylorus, the function of *Gata3* was unclear when I began my studies. However, it appears to be expressed in the pyloric mesenchyme and is inhibited by ectopic pyloric expression of canonical WNTs [109]. The work presented in this thesis will expand on the current understanding of the expression and function of *Gata3* at the mouse pylorus.

### 1.6.3 Conflicts between the mouse and chick pyloric signaling models

As I summarized above, the expression domains of several pyloric transcription factors and signaling molecules are well known in both the mammalian and avian models [78-80, 102, 106]. However, these models have demonstrated different, even conflicting results in pyloric signaling [78-80]. Specifically, in the chick pylorus, BMP has been shown to signal downstream to *Nkx2-5* and *Sox9* [5, 78], which both regulate the character of pyloric epithelial microarchitecture [78].

In addition, in the chick pylorus, *Sox9* appears to perform its function through the BMP signaling modulator *Grem1*, which also regulates pyloric epithelial morphogenesis [78]. In contrast, the mouse *Six2* knockout model appears to show that BMP, *Nkx2-5* and *Sox9* are altered in opposite directions, with loss of *Six2* leading to increased *Bmp4* expression but decreased *Nkx2-5* and *Sox9* expression. While it may be that *Six2* regulation of these genes is complex and interconnected, it may also be that gene expression and function at the chick pylorus differs from that at the mouse pylorus. The work presented in Chapter 2 will clarify the relationship between *Nkx2-5* and *Sox9* and establish the function of *Nkx2-5* in pyloric development.

### 1.7 Expanding the State of the Field

Though it is clear that multiple proteins and signaling factors are needed for the proper formation of the pylorus, it is striking that the vast majority of the published work used either Q-RT-PCR or whole mount *in situ* hybridization to follow the expression of genes after deletion or overexpression of candidate regulators. Thus, it had not been determined which of the multiple factors are actually expressed in the same cell. Indeed, even the identities of the cells that express each transcription factor were unknown at the time that I started these studies. Additionally, as pointed out above, there were major discordancies among the various published studies both with regard to the actual signaling cascades required for pyloric development and with regard to the function of each factor (e.g., regulation of epithelial character or muscular development). My goal in this thesis was first to clarify both the expression and the function of some of the major regulators of pyloric development. *Nkx2-5* was prioritized because of its genetic association with IHPS [13] and *Gata3* was prioritized because it was a novel pyloric-specific gene first identified by studies from our laboratory [7]. In the following chapters, I will present data that clarifies pyloric development and shows for the first time that a specific fascicle of outer longitudinal muscle can play a major role in pyloric sphincter development. I will also explain how these findings extend our current understanding of both the pyloric signaling cascade and the etiologies of the pyloric pathologies DGR and IHPS.

Establishment of the domains of both *Gata3* and *Nkx2-5* expression led to a serendipitous finding that I pursued in Chapter 3. Specifically, I found that both proteins are expressed in a pair of bilateral superficial cords on the ventral surface of the stomach; these structures had been previously defined as gastric ligaments. Further investigation revealed that they are smooth muscle structures that are continuous with the outer longitudinal muscle. Chapter 3 provides the first molecular and cellular characterization of these structures, which we have renamed “pyloric ligaments”. Finally, in Chapter 4, I will conclude with an examination of the open questions remaining within the field, particularly those that arise as a consequence of this work, and will propose further experimentation to expand upon the presented work.

# Chapter 2 [1]

## The role of Gata3 and Nkx2-5 in pyloric development

### Summary

Background & Aims: Infantile hypertrophic pyloric stenosis (IHPS) is a common birth anomaly characterized by narrowing of the pyloric lumen. A recent genome wide association study implicated *NKX2-5*, a gene encoding a transcription factor that is expressed in embryonic heart and pylorus, in the pathogenesis of IHPS. However, the function of the NKX2-5 protein in pyloric smooth muscle development has not been directly examined. Hence, our goals were to: a) define the pattern of *Nkx2-5* expression during the course of murine pyloric sphincter development; b) examine co-expression of *Nkx2-5* with *Gata3* and *Sox9*, two other transcription factor genes that display pyloric-specific mesenchymal expression domains; and, c) assess pyloric sphincter development in the absence of *Nkx2-5* or *Gata3*.

Methods: Cellular domains of NKX2-5, GATA3, and SOX9 expression were determined by immunofluorescence. Pyloric development was assessed in mice with conditional or germline deletion of *Nkx2-5* or *Gata3*, respectively.

Results: *Gata3, Nkx2-5*, and *Sox9* are co-expressed in differentiating smooth muscle cells of a distinct fascicle of the pyloric outer longitudinal muscle (OLM). Expansion of this fascicle

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[1] This text is from a submitted manuscript. The contributing authors are: Aaron M. Udager*, Ajay Prakash*, David A. Saenz, Martina Schinke, Takashi Moriguchi, Patrick Y. Jay, Kim-Chew Lim, James Douglas Engel, and Deborah L. Gumucio [The first two co-authors made equal contributions to the work.]

coincides with development of the pyloric sphincter. Absence of *Nkx2-5* or *Gata3* causes severe hypoplasia of this fascicle and alters pyloric muscle shape. While *Sox9* expression requires both *Nkx2-5* and *Gata3*, there is no apparent hierarchical relationship between *Nkx2-5* and *Gata3* during pyloric OLM development.

<u>Conclusions</u>: *Nkx2-5* and *Gata3* are independently required for the development of a pyloric OLM fascicle that plays a critical role in pyloric sphincter morphogenesis. These data predict that regulatory changes that alter *Nkx2-5* or *Gata3* expression could play a role in IHPS pathogenesis.

## 2.1 Introduction

The pyloric sphincter integrates neuronal and hormonal signals to control the movement of food from the stomach to the small intestine [118]. This sphincter is clinically significant in the context of the common human congenital pathology, infantile hypertrophic pyloric stenosis (IHPS), in which both the structure and function of the sphincter are abnormal[9, 24, 119]. Infants with IHPS classically present three to six weeks after birth with projectile vomiting, as well as physical and radiographic findings of gastric outlet obstruction. The etiology of IHPS appears to be complex and may involve both environmental and genetic factors; changes in musculature, mucosa, extracellular matrix, nerve conduction, and nitric oxide signaling have all been implicated in IHPS pathogenesis[9, 24, 119].

A recent genome-wide association study (GWAS) in humans identified several IHPS susceptibility loci, including the homeodomain transcription factor *NKX2-5[13]*. This is of interest because *Nkx2-5* is expressed in pyloric mesenchyme during embryogenesis in frog, chick, and mouse[1], although the precise identity of the expressing cells is unknown. Despite its evolutionarily conserved pyloric expression and association with IHPS, the role of *Nkx2-5* in pyloric development has not been examined in vertebrate models, in part because *Nkx2-5* null mice die of cardiac abnormalities at E10[120], well before the pyloric region is fully developed.

Work in the chick model suggests that BMP signaling controls the expression of both *Nkx2-5* and the SRY-related, HMG-box gene *Sox9[5, 78, 79, 102]*. Functionally, loss of either *Nkx2-5* or *Sox9* expression in the chick affects the character of the pyloric epithelium but has no effect on the pyloric musculature[5, 78, 79, 102], suggesting that these mesenchymal factors act indirectly to control the expression of an unknown modulator of epithelial phenotype.

In the mouse, direct functional analysis of *Nkx2-5* or *Sox9* at the pylorus has not been reported; however, other genetic models of pyloric sphincter dysmorphogenesis have been described[80, 105, 107-109]. For example, germline deficiency of *Six2*, a homeodomain transcription factor expressed in the posterior stomach, abrogates *Sox9* expression and temporarily reduces *Nkx2-5* expression at the pylorus, though this expression is later recovered[80]. Importantly, in *Six2* mutant mice, the pyloric musculature and its corresponding luminal constriction are highly attenuated, indicating that *Six2* and/or one or more of its downstream targets is important for pyloric sphincter development.

Though a role for *Nkx2-5* in the formation of the pyloric sphincter might be inferred from the phenotype of *Six2* null mice, a direct connection between *Nkx2-5* and sphincter muscle development has not been demonstrated in either the mouse or chick models. In fact, while it is clear from previous studies that *Nkx2-5* is expressed in pyloric mesenchyme, we present here the first analysis of its expression at the cellular level during pyloric sphincter development and correlate this expression pattern with development of the sphincter muscles.

We find that NKX2-5 protein is expressed in myofibroblasts and smooth muscle cells of the pylorus. NKX2-5 expression is most robust in a dorsal fascicle of outer longitudinal muscle (OLM) that matures between embryonic days (E) 14.5 and 16.5. Interestingly, the cells of this OLM fascicle also express SOX9, as well as GATA3, a zinc finger transcription factor that we previously identified as a pylorus-specific gene[7]. After germline deletion of *Gata3* or conditional deletion of *Nkx2-5,* the dorsal pyloric OLM fascicle is hypoplastic, the shape of the inner circular muscle (ICM) is altered and constriction of the pyloric sphincter is attenuated. Together, these data reveal a distinct transcriptional regulatory cascade that is used for development of the dorsal pyloric OLM; correct development of this fascicle is required to generate the proper morphology of the pyloric sphincter. These findings have implications for the potential role of *NKX2-5* in the pathogenesis of IHPS in humans.

## 2.2 Materials and methods

### *Mice*

All protocols for mouse experiments were approved by and carried out in accordance with the policies of the University of Michigan University Committee of Use and Care of Animals and Unit for Laboratory Animal Medicine. C57BL/6J inbred ("wild type") mice were obtained from

Charles River Laboratories (Wilmington, MA). The generation of *Gata3*$^{lacZ/+}$, *Nkx2-5*$^{lacZ/+}$ and *CAGGCre-ER*$^{TM}$ mice has been described previously[117, 121, 122].

*Gata3* null embryos were generated via *Gata3*$^{lacZ/+}$ intercrosses. To escape early embryonic lethality, *Gata3* null embryos were pharmacologically rescued *in utero* by treating timed-pregnant dams with α- and β-adrenergic agonists, as previously described[110, 113]. The rescue solution was administered once daily, beginning at E7.5, and all other drinking water was withheld. Rescue solution was prepared fresh, as follows: 15 mg each of isoproterenol (Sigma-Aldrich, St. Louis, MO, I-5627) and phenylephrine (Sigma-Aldrich, P-6126) was added to 50 mL of water and supplemented with 100 mg of ascorbic acid and 2 g of sucrose.

*Nkx2-5*$^{flox/+}$ mice were generated by targeted homologous recombination in embryonic stem cells, as described previously[123, 124]. The targeting construct was created by cloning a neomycin resistance cassette, flanked by FLP recognition target (FRT) sites, into intron 1 of *Nkx2-5*; loxP sites were then cloned upstream of the neomycin resistance cassette and downstream of the homeodomain-containing exon 2 (Figure 2.1B). The neomycin resistance cassette was excised via FLP-mediated FRT site recombination, resulting in loxP sites flanking exon 2 (Figure 2.1C). *Nkx2-5*$^{flox/+}$ mice were crossed to *CAGGCre-ER*$^{TM}$ (The Jackson Laboratory, Bar Harbor, ME, 004682) and bred to homozygosity for the conditional allele (*CAGGCre-ER*$^{TM}$;*Nkx2-5*$^{flox/flox}$). Inactivation of *Nkx2-5* in timed-pregnant dams was accomplished via once-daily intraperitoneal injections of tamoxifen (3 mg in 150 μL of corn oil; Sigma-Aldrich, T5648), as described previously[121].

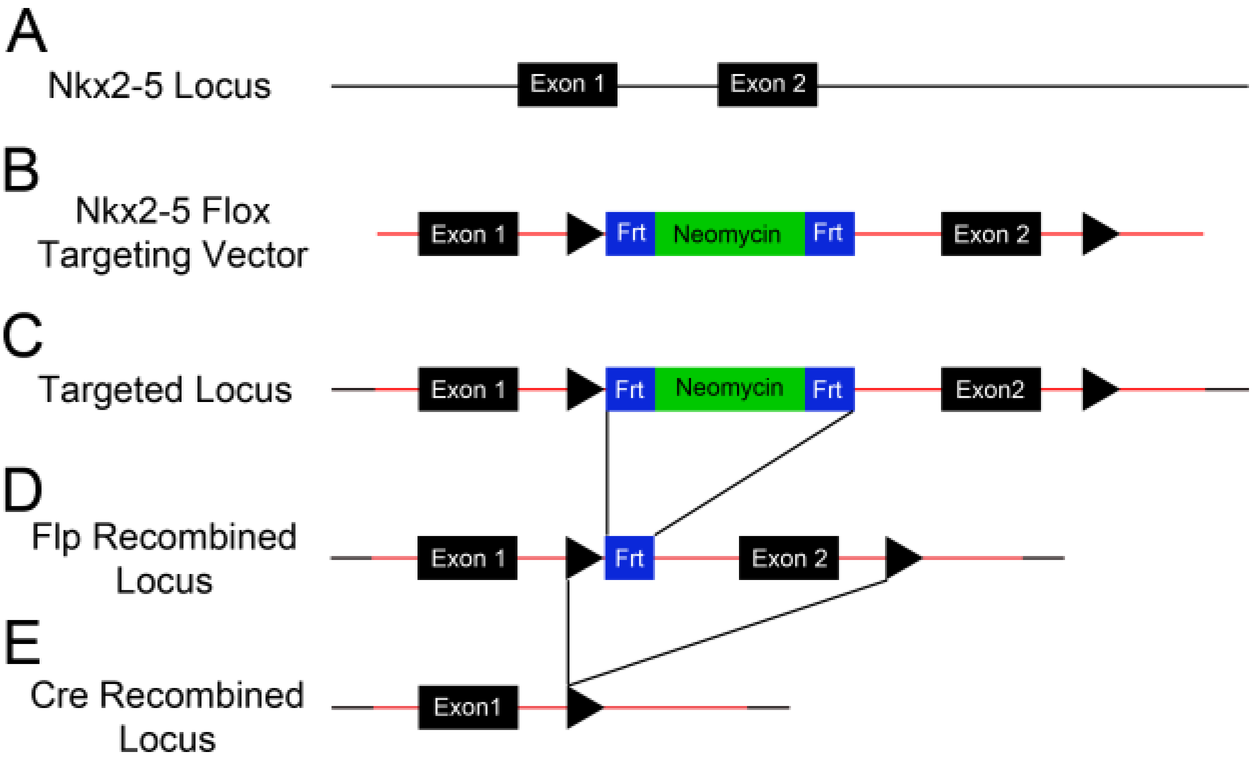


*Figure 2.1 Generation of a conditional Nkx2-5 allele.*
Illustration of the gene targeting approach used to produce a conditional Nkx2-5 allele. A) The wild type Nkx2-5 locus contains two exons. B) The targeting vector contains a neomycin selection cassette with flanking FRT sites in intron 1; flanking 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 (Nkx2-5flox), which has loxP sites flanking exon 2 and a single FRT site in intron 1 ("Flp Recombined Locus"). In cells that express Cre and Nkx2-5flox, 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 2.11Biii).

### *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.

### *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). 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).

### *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 H&E, 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). 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, who counted the number of discrete fluorescent signals (Tables 2 and 3). Cell number and percentage of total cells were analyzed by the Student's t-test statistic in Excel (Microsoft, Redmond, WA; unpaired, heteroscedastic).

### *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). 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 Gata3lacZ/+ or Nkx2-5lacZ/+ 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.

## 2.3 Results

### *Development of pyloric muscular components*

Despite the important function of pyloric sphincter, development of its smooth muscle components has not been assessed at the cellular level. We therefore examined sectioned pyloric tissue using H&E staining and immunofluorescence for alpha smooth muscle actin (αSMA), a marker of differentiated smooth muscle cells and myofibroblasts. At E14.5, the ICM at the pylorus is contiguous with that of the surrounding stomach and intestine and strongly expresses αSMA (Figure 2.2A-B). In contrast, the nascent OLM contains a thin layer of weakly positive αSMA positive cells (Figure 2.2Bv,vi, asterisk). These cells are not yet tightly organized into muscular bundles. Dorsally, these cells bridge directly to and intermingle with a prominent collection of αSMA negative pancreatic mesenchymal cells (Figure 2.2Aii, PM).

By E16.5, the pyloric OLM is compacted and robustly expresses αSMA, indicative of smooth muscle differentiation (Figure 2.2C-D). Dorsally, a thickened fascicle of OLM appears to displace the ICM internally (Figure 2.2Dv,vi, asterisk), thereby narrowing the pyloric lumen to generate the characteristic constriction of the mature pyloric sphincter.

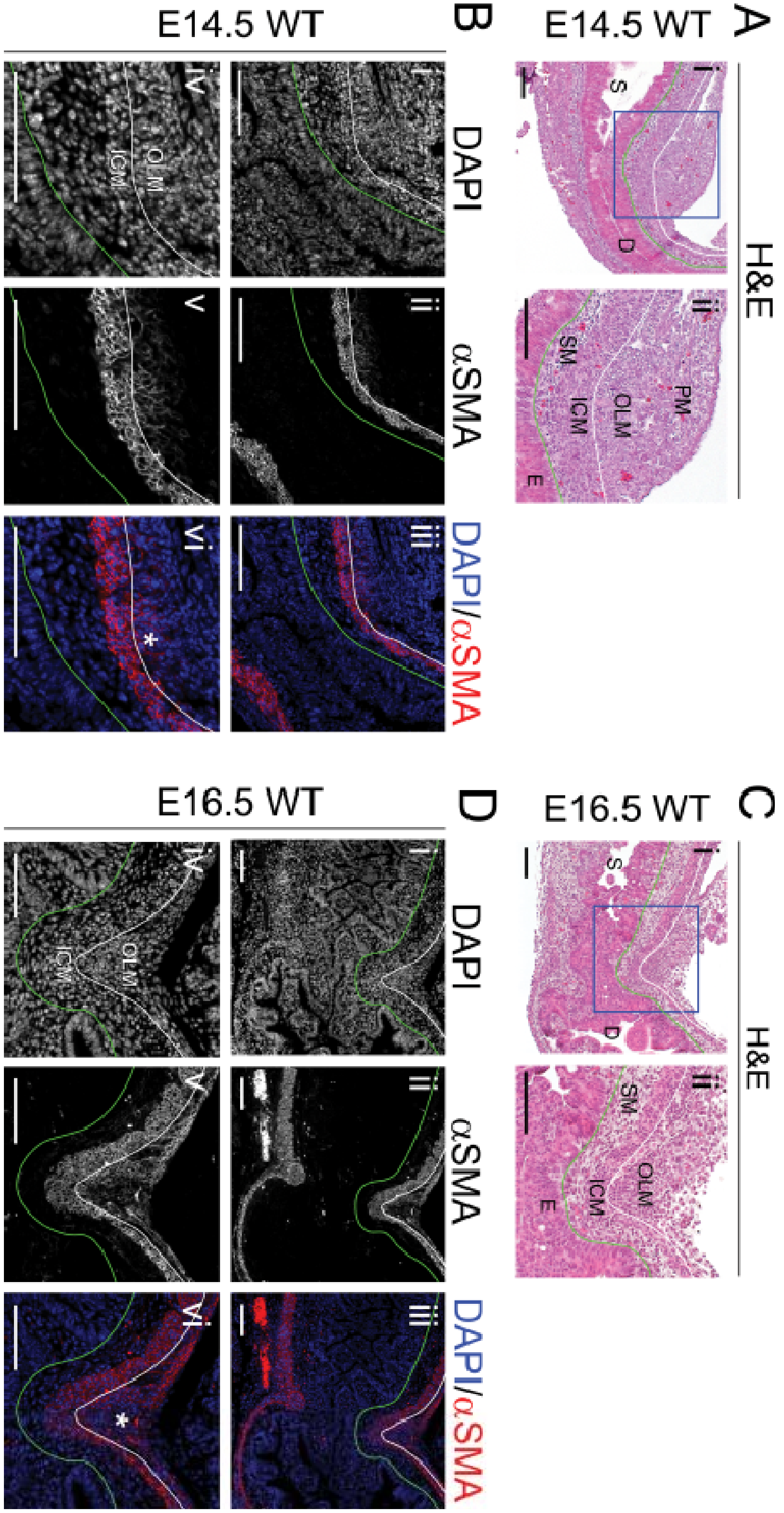

A
E14.5 WT
H&E
i
S
D
ii
PM
OLM
ICM
SM
E
B
E14.5 WT
DAPI
αSMA
DAPI/αSMA
i
ii
iii
iv
OLM
ICM
v
vi
*
C
E16.5 WT
H&E
i
S
D
ii
SM
OLM
ICM
E
D
E16.5 WT
DAPI
αSMA
DAPI/αSMA
i
ii
iii
iv
OLM
ICM
v
vi
*

*Figure 2.2 Development of the pylorus between E14.5 and E16.5 involves differentiation of the dorsal OLM and changes in ICM shape.*

H&E staining of WT pylorus at (A) E14.5 or (C) E16.5; the dorsal pylorus, within the boxed region in (i) is enlarged in (ii). Immunofluorescence of the dorsal WT pylorus at (B) E14.5 or (D) E16.5: (i,iv) DAPI; (ii,v) αSMA; or (iii,vi) merged. (A-B) Though the ICM is well-differentiated and strongly αSMA positive at E14.5, cells of the OLM stain weakly for αSMA (asterisk in Bvi). (A-B) Expansion and differentiation of the OLM (asterisk in Dvi) is associated with an increase in αSMA expression and inward displacement of the ICM, resulting in pyloric sphincter constriction. For all images, stomach (S) is left; duodenum (D) is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. E = epithelium; SM = sub-epithelial mesenchyme; and, PM = pancreatic mesenchyme. Scale bars represent 100 μm.

### *Nkx2-5 and Gata3 are expressed in similar domains at the pylorus*

Previous studies have shown that, as early as E9.5, a mesenchymal Nkx2-5 expression domain surrounds the nascent distal stomach and proximal duodenal endoderm[25]. By E12.5, some cells within this mesenchymal domain have migrated anteriorly along the left (dorsal) side of the stomach to give rise to the spleen, while others remain at the pylorus[25]. Since the late embryonic expression pattern of *Nkx2-5* at the pylorus has not been carefully described, we examined whole mount X-gal staining of dissected tissue from *Nkx2-5*$^{lacZ/+}$ embryos. At E14.5, *Nkx2-5*-expressing cells encircle the pylorus and staining on the dorsal side of the pylorus extends into adjoining pancreatic mesenchyme (Figure 2.3Ai). Bilateral cellular cords, emanating from the pylorus and reaching across the lesser curvature of the antrum, also express *Nkx2-5* (Figure 2.3Ai, black arrowheads); these structures likely correspond to the previously described gastric ligaments[125]. Continuity between the X-gal positive pyloric band and gastric ligaments is obvious ventrally (Figure 2.3Aii). Between E14.5 and E18.5, the gastric ligaments lengthen to reach the gastro-esophageal junction, but the *Nkx2-5* expression pattern is otherwise unchanged (Figure 2.3Aiii).

In a previous study, we found that *Gata3* exhibits a pyloric-specific expression domain in the mesenchyme, similar to that of *Nkx2-5[7]*. To further characterize *Gata3* expression at the pylorus, we examined dissected whole mount X-gal stained tissue from *Gata3*$^{lacZ/+}$ embryos. Similar to *Nkx2-5*, *Gata3* is expressed in a discrete band at the pylorus, as well as in the gastric ligaments (Figure 2.3Bi, black arrowheads). Notably, the pyloric *Gata3* expression domain is

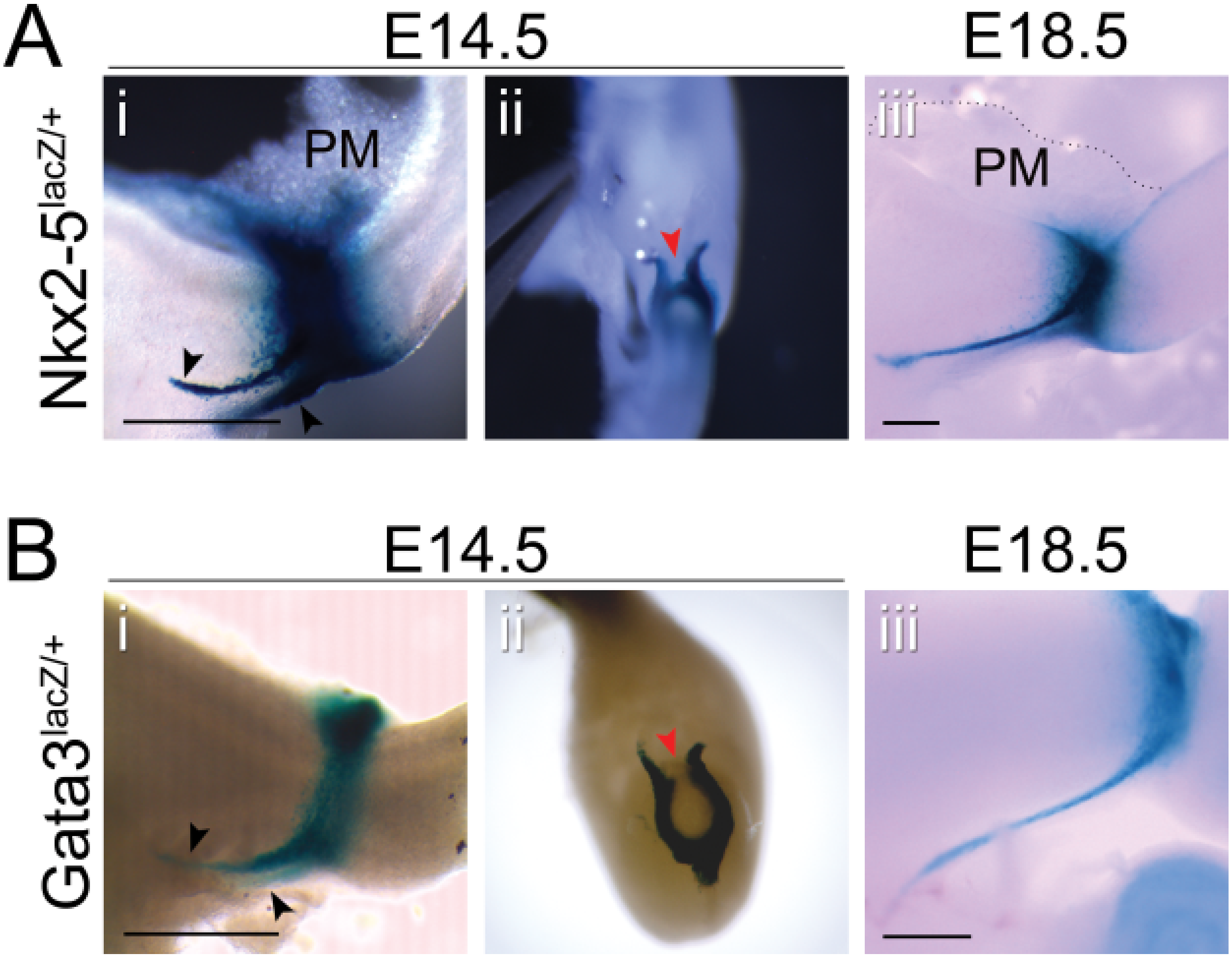


*Figure 2.3 Gata3 and Nkx2-5 are expressed in similar domains at the pylorus.*
Whole mount X-gal staining of pylorus from (A) Nkx2-5lacZ/+ or (B) Gata3lacZ/+ mice at (i-ii) E14.5 and (iii) E18.5. (i,iii) Lateral view: stomach is left; duodenum is right; dorsal is top. (ii) Ventral view: stomach is top; duodenum is bottom. (Ai,Bi) Nkx2-5 and Gata3 have similar expression patterns, with extension dorsally into the pancreatic mesenchyme (PM in Aii) and ventrally into the gastric ligaments (black arrowheads in Ai and Bi), however, the width of the Gata3 expression domain is narrower than Nkx2-5. (Aii,Bii) Nkx2-5 expression is completely circumferential (red arrowhead in Aii), while Gata3 expression is discontinuous ventrally (red arrowhead in Bii). (Aiii,Biii) By E18.5, the gastric ligaments have lengthened to reach the esophagus (pancreatic mesenchyme outlined by dashed line in Aiii). Scale bars = 100 µm.

narrower than *Nkx2-5*, and on the lesser curvature (ventral) side, there is a small gap in the *Gata3* expression domain (Figure 2.3Bii, red arrowhead).

### Co-expression of pyloric transcription factors during pyloric sphincter maturation

Self et al. previously showed that, between E12.5 and E14.5, Nkx2-5 and Sox9 are expressed in similar domains at the murine pylorus[16]. This is important since both of these genes have been implicated in pyloric development[8-11]. However, we were interested to: a) determine whether *Nkx2-5* and *Sox9* are co-expressed in the same cells; b) compare these domains with the expression pattern of *Gata3*; and, c) examine expression of these genes during establishment of the complete pyloric musculature (E14.5 to E16.5).

The results of this expression analysis reveal distinct pyloric cell populations that are single, double, or triple positive for NKX2-5, SOX9, and/or GATA3, suggesting that a complex developmental circuitry directs pyloric development. At both E14.5 and E16.5, the expression domains of GATA3 and NKX2-5 are exclusively mesenchymal, while SOX9 is expressed in both epithelium and mesenchyme, as previously noted (Figure 2.5)[80]. A line of enteric neurons that are strongly positive for SOX9 and the neuronal marker peripherin, but negative for GATA3 and NKX2-5 (Figure 2.4), separates the OLM and ICM domains (white lines in Figure 2.5). At E14.5, cells that are exclusively NKX2-5 positive are detected in the sub-epithelial mesenchyme and in the ICM (Figure 2.5Aviii, yellow arrowheads), while cells that express only GATA3 are located in the outermost dorsal pylorus, where it mixes with pancreatic mesenchyme (Figure 2.5Aix, white arrowheads). Some of these GATA3 positive cells are epithelial in nature and may be part of the developing serosa (Figure 2.6).

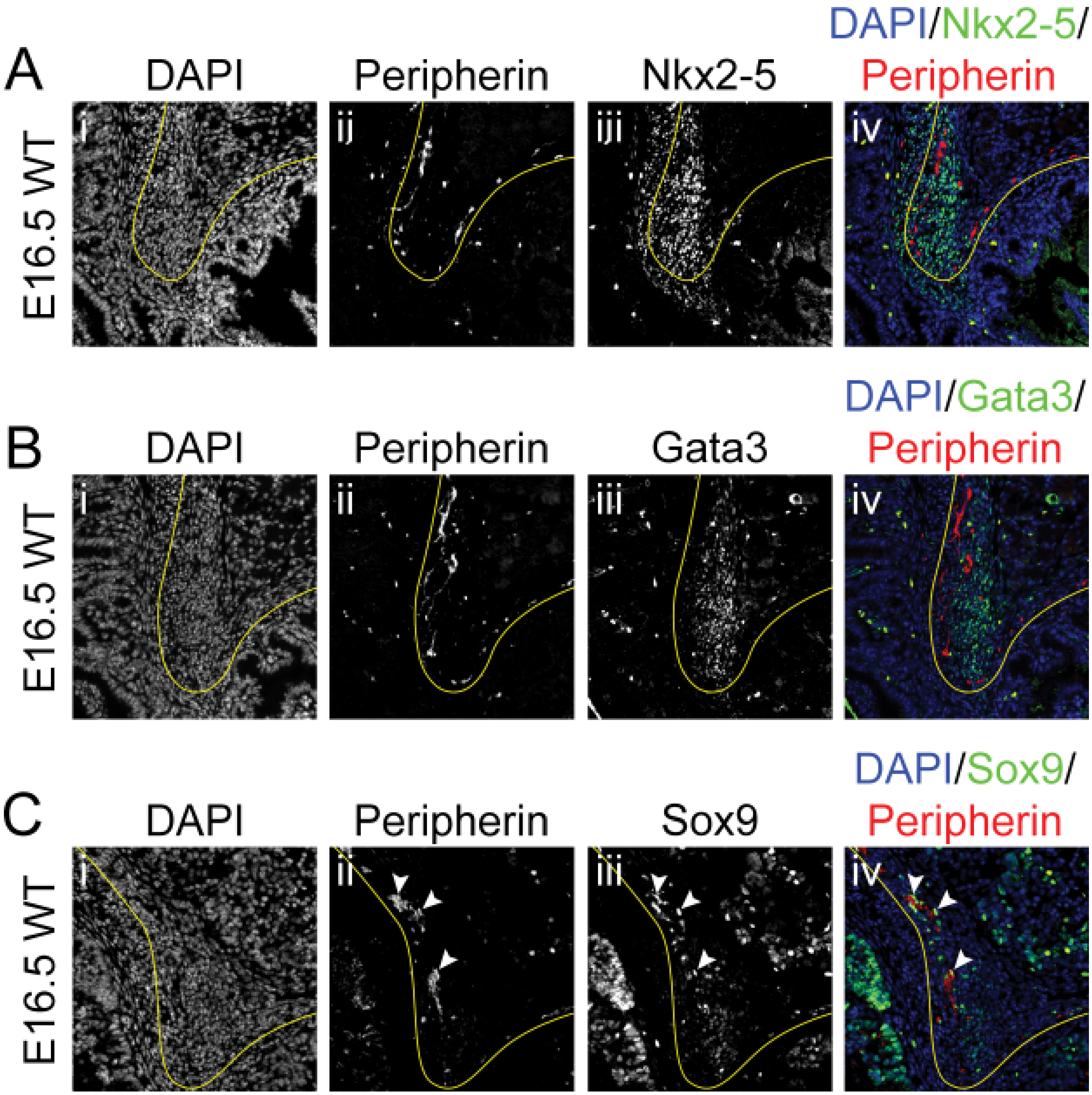
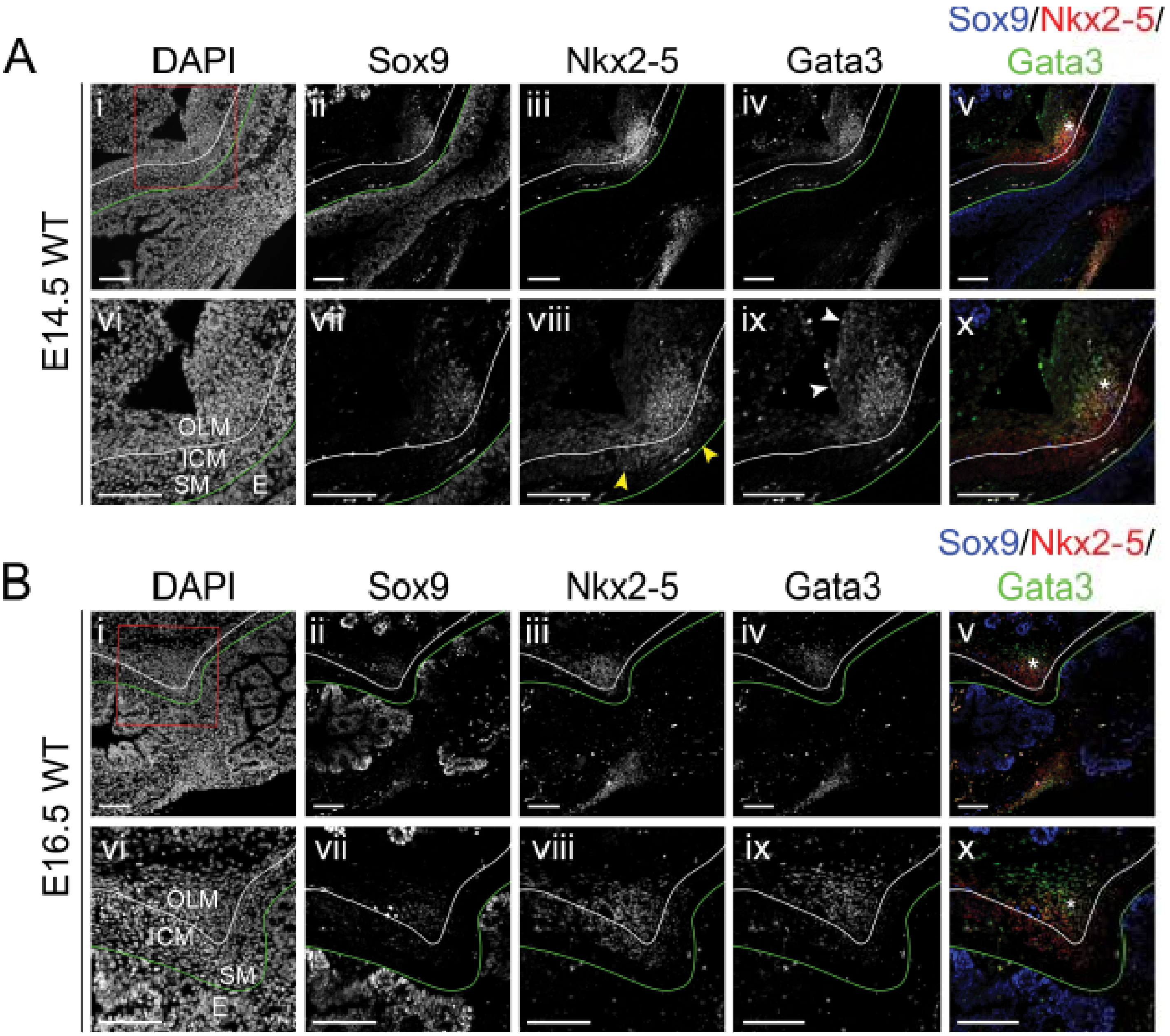


*Figure 2.4 Enteric neurons express SOX9 but not NKX2-5 or GATA3.*
Immunofluorescence of WT pylorus at E16.5: (i) DAPI; (ii) peripherin; (Aiii) NKX2-5; (Biii) GATA3; (Ciii) SOX9; or (iv) merged. (A-C) Enteric neurons express SOX9 (arrowheads in Civ) but not NKX2-5 or GATA3. Stomach is left; duodenum is right; and, dorsal is top. A dotted line is placed just beneath the line of peripherin positive neurons that separate ICM from OLM.


A
DAPI
Sox9
Nkx2-5
Gata3
Sox9/Nkx2-5/
Gata3
E14.5 WT
i
ii
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OLM
ICM
SM
E
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DAPI
Sox9
Nkx2-5
Gata3
Sox9/Nkx2-5/
Gata3
E16.5 WT
i
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vii
viii
ix
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OLM
ICM
SM
E
*

*Figure 2.5 NKX2-5, GATA3, and SOX9 are co-expressed in dorsal pyloric OLM.* Immunofluorescence of WT pylorus at (A) E14.5 or (B) E16.5 : (i,iv) DAPI; (ii,vii) SOX9; (iii,viii) NKX2-5; (iv,ix) GATA3; or (v,x) merged. (A-B) NKX2-5 single positive cells are found in the sub-epithelial mesenchyme and ICM (yellow arrowheads in Aviii), while GATA3 single positive cells are present in the pancreatic mesenchyme (white arrowheads in Aix). NKX2-5, GATA3, and SOX9 are co-expressed in the dorsal OLM (asterisks in Av,x and Bv,x). Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. SM = sub-epithelial mesenchyme. Scale bars = 100 µm.

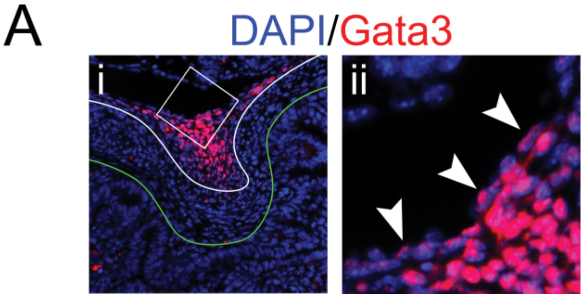


*Figure 2.6 GATA3 positive cells contribute to the serosal lining at the pylorus.*
GATA3 immunofluorescence of WT pylorus at E16.5: (i-ii) merged DAPI (blue) and GATA3 (red) images; the dorsal pylorus within the boxed region in (i) is enlarged in (ii). GATA3 is expressed in the dorsal OLM and scattered epithelial 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.

Cells that are triple positive for NKX2-5, GATA3, and SOX9 are visible at E14.5 as a loose cluster of cells located primarily within the nascent OLM territory on the dorsal side (Figure 2.5Av,x, asterisk). Comparison with Figure 2.2 indicates that some of these cells are weakly αSMA positive (Figure 2.2Bvi, asterisk). Two days later, these OLM cells continue to express all three transcription factors (Figure 2.5Bv,x, asterisk) and now also strongly express αSMA (Figure 1Dvi, asterisk). Thus, cells that co-express all three transcription factors appear to contribute primarily to the OLM.

*Nkx2-5 is required for development and maintenance of the pyloric OLM*

The expression pattern of Nkx2-5 suggests a potential role for this transcription factor in pyloric development. However, germline deficiency of Nkx2-5 results in embryonic lethality by E10[7], precluding analysis of Nkx2-5 function in late embryogenesis. Thus, we paired a conditional Nkx2-5 allele (Nkx2-5flox/flox) with a tamoxifen-inducible Cre recombinase transgene driven from a ubiquitously expressed transgenic promoter (CAGGCre-ERTM)[19]. Two cohorts of pregnant dams were treated with once-daily tamoxifen injections, beginning at E14.5 or E16.5; each cohort was sacrificed two days later for analysis.

Deletion of Nkx2-5 beginning at E14.5 leads to nearly complete absence of the αSMA positive pyloric OLM smooth muscle at E16.5 (Figure 2.7Av,vi, asterisks), suggesting that Nkx2-5 is necessary for the maturation of the OLM. Interestingly, Nkx-5 deletion beginning at E16.5, when the OLM is already formed and strongly expresses αSMA (Figure 2.2Dv,vi), significantly reduces OLM smooth muscle by E18.5 (Figure 2.7Bv,vi, asterisks). Remaining cells are loosely organized and weakly rather than strongly αSMA positive, suggesting that Nkx2-5 function is

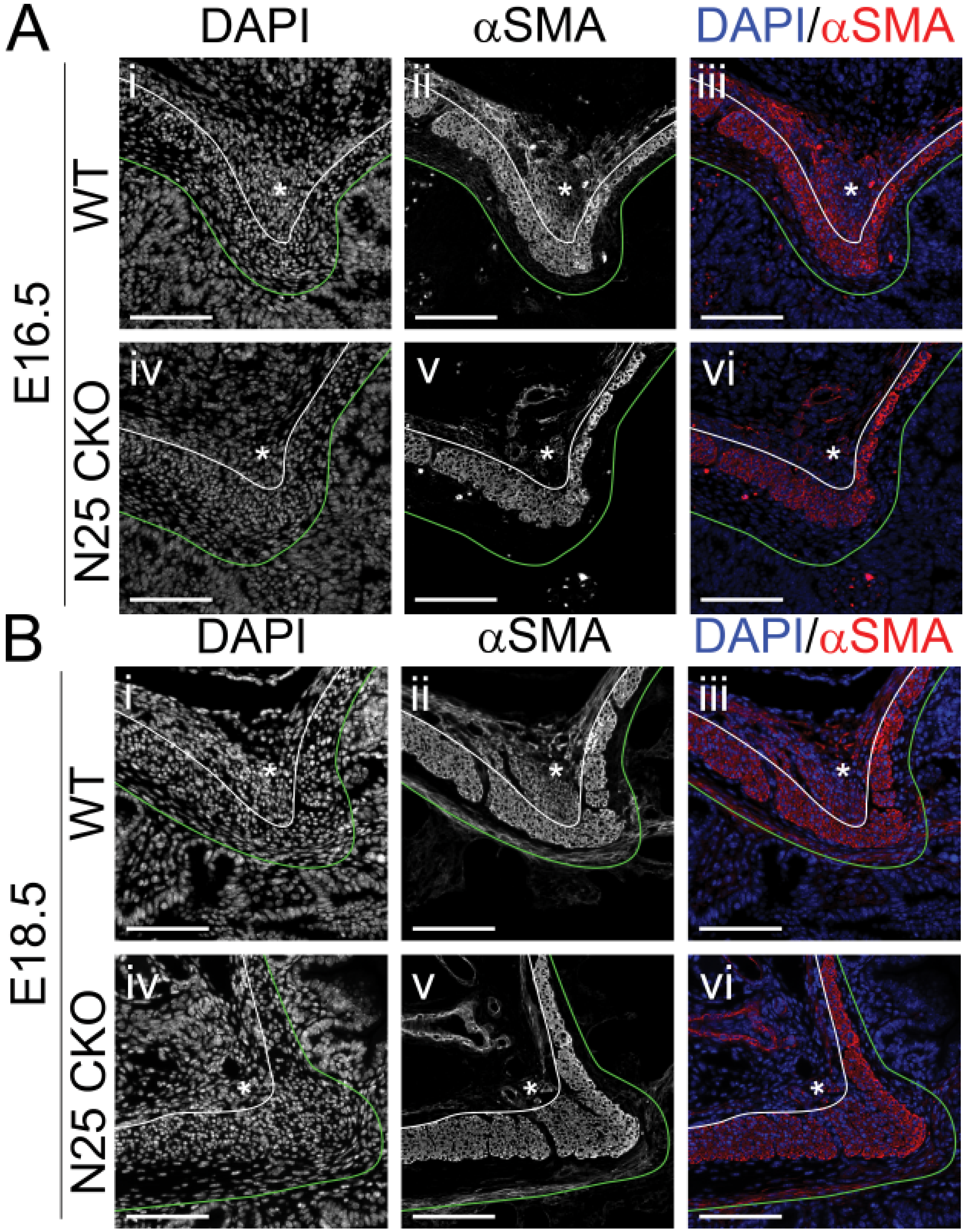

A
DAPI
αSMA
DAPI/αSMA
E16.5
WT
N25 CKO
i
ii
iii
iv
v
vi
*
B
DAPI
αSMA
DAPI/αSMA
E18.5
WT
N25 CKO
i
ii
iii
iv
v
vi
*

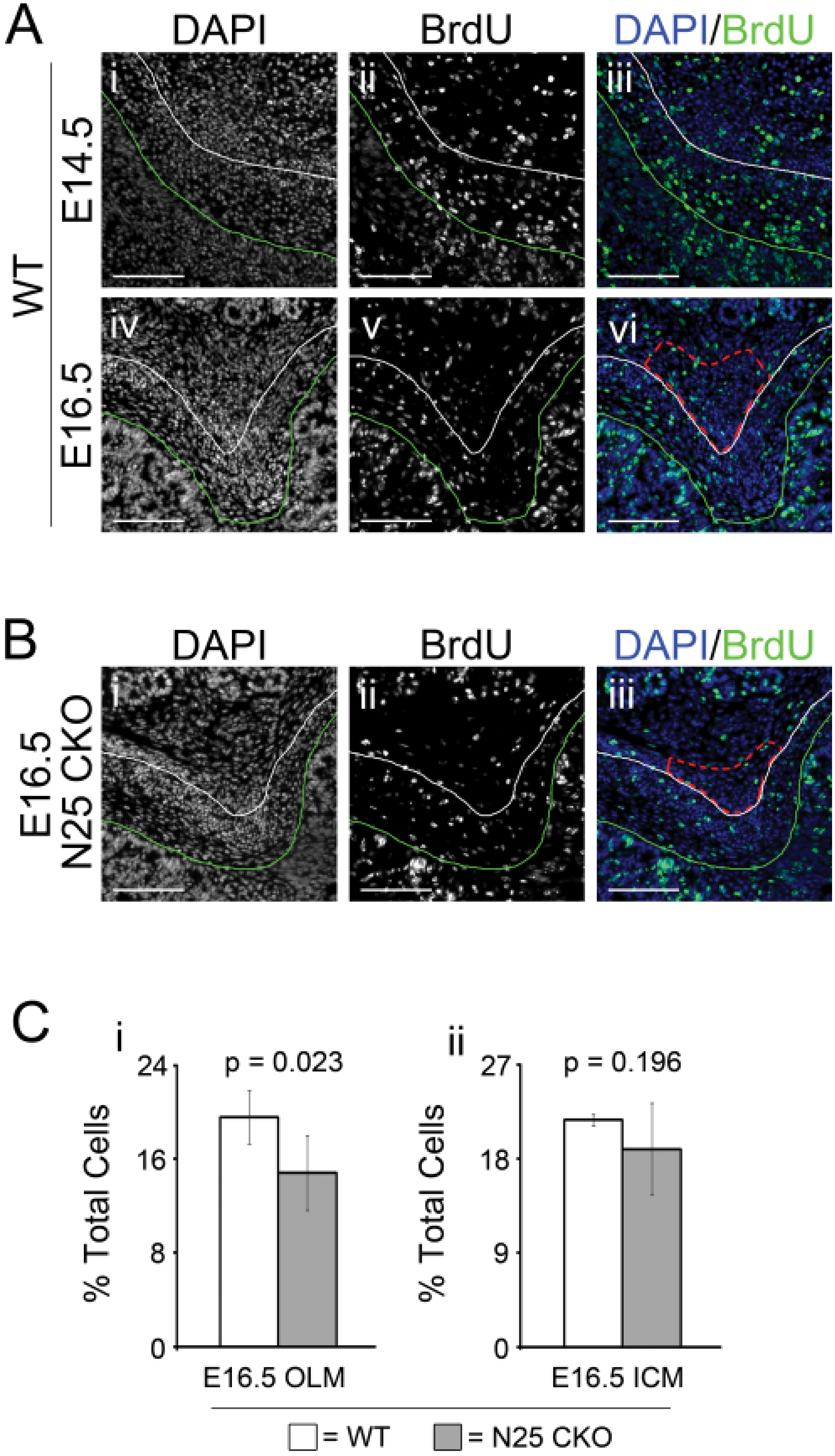
*Figure 2.7 Nkx2-5 is required for the development and maintenance of the dorsal pyloric OLM.* Immunofluorescence of (i-iii) Nkx2-5flox/flox (WT) or (iv-vi) CAGGCre-ERTM;Nkx2-5flox/flox (N25 CKO) pylorus (after two days of intraperitoneal tamoxifen injections) harvested at (A) E16.5 or (B) E18.5: (i,iv) DAPI; (ii,v) αSMA; or (iii,vi) merged. (A) In N25 CKO mice at E16.5, cell mass and αSMA□expression is reduced in the dorsal OLM (asterisks). (B) At E18.5, the dorsal OLM in WT mice has expanded further and shows increased αSMA expression (Bi-iii). Deletion of Nkx2-5 beginning at E16.5, when the dorsal OLM is already αSMA□positive (Aii-iii), leads to its regression. Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. Asterisk = dorsal pyloric OLM. Scale bars = 100 µm.

continuously required for the maintenance of differentiated smooth muscle cells within this fascicle.

### *Increased apoptosis and reduced proliferation in pyloric OLM after loss of Nkx2-5*

Our data show that loss of Nkx2-5 beginning at E14.5 prevents the maturation of the pyloric OLM fascicle, and deletion of this gene after the fascicle has formed at E16.5 leads to its regression. Reduced smooth muscle cell proliferation and/or increased apoptosis are potential mechanisms underlying these OLM responses. To examine proliferative changes, timed-pregnant dams were injected with BrdU two hours prior to sacrifice. In wild type (WT) animals, BrdU positive cells were found scattered throughout the ICM and OLM regions at E14.5 and E16.5 (Figure 2.8A). Conditional deletion of *Nkx2-5* beginning at E14.5 was associated with a 25% reduction in the proportion of proliferative cells in the OLM fascicle at E16.5 (19.6% in WT versus 14.8% after *Nkx2-5* deletion, $P < 0.05$) (Figure 2.8B, Ci). Though *Nkx2-5* is also expressed in the ICM, no significant change in proliferative activity was detectable in that domain (Figure 2.8Cii).

To assess the impact on apoptosis, we examined Caspase3 (Casp3) expression 24 and 48 hours after deletion of *Nkx2-5.* At 48 hours post-deletion, the OLM fascicle was largely absent, and the remaining cells were not Casp3 positive (data not shown). At 24 hours post-deletion, though the total number of cells (as well as the number of Nkx2-5 positive cells) had already decreased, the proportion of Casp3 positive cells within the OLM had nearly doubled (2.6% in WT versus 4.8% after *Nkx2-5* deletion, $P = 0.07$) (Figure 2.9). We conclude that deletion of *Nkx2-5* results in decreased proliferation, increased apoptotic activity and rapid loss of the dorsal pyloric OLM


A
DAPI
BrdU
DAPI/BrdU
WT
E14.5
E16.5
i
ii
iii
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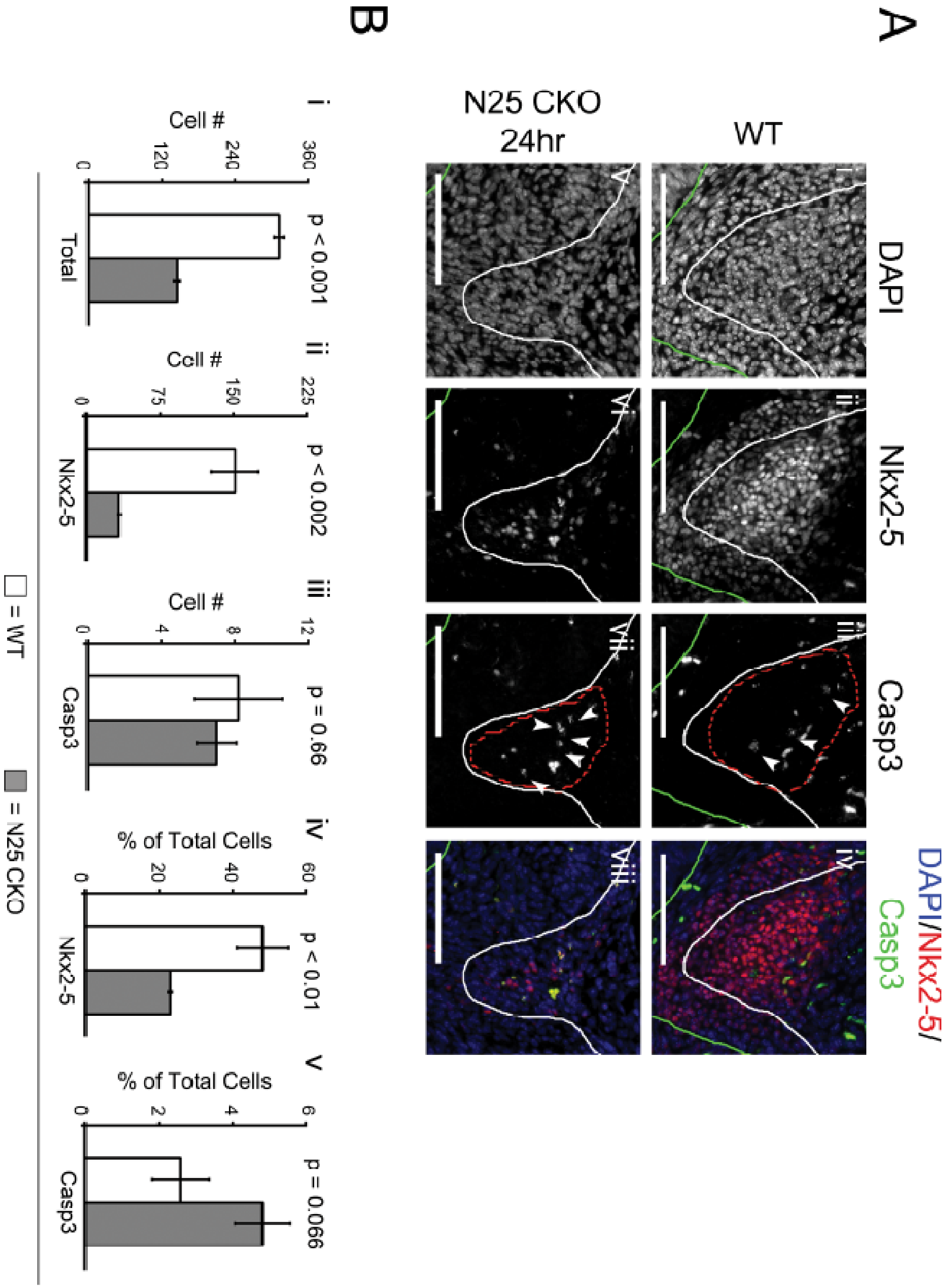
vi
B
DAPI
BrdU
DAPI/BrdU
E16.5
N25 CKO
i
ii
iii
C
i
p = 0.023
% Total Cells
24
16
8
0
E16.5 OLM
ii
p = 0.196
% Total Cells
27
18
9
0
E16.5 ICM
□ = WT
▨ = N25 CKO

*Figure 2.8 Decreased cellular proliferation in the pyloric OLM after Nkx2-5 deletion.* Immunofluorescence of (A) WT or (B) 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.



A
WT
N25 CKO 24hr
DAPI
Nkx2-5
Casp3
DAPI/Nkx2-5/Casp3
i
ii
iii
iv
v
vi
vii
viii
B
i
Cell #
0
120
240
360
p < 0.001
Total
ii
Cell #
0
75
150
225
p < 0.002
Nkx2-5
iii
Cell #
0
4
8
12
p = 0.66
Casp3
iv
% of Total Cells
0
20
40
60
p < 0.01
Nkx2-5
v
% of Total Cells
0
2
4
6
p = 0.066
Casp3
☐ = WT
▨ = N25 CKO

*Figure 2.9 Increased cellular apoptosis in the pyloric OLM after Nkx2-5 deletion.*
(A) Immunofluorescence of (i-iv) WT or (v-viii) N25 CKO pylorus (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

fascicle. Interestingly, though the ICM also expresses *Nkx2-5*, proliferation and apoptosis within this domain does not appear to be altered, indicating that a distinct molecular network directs the maturation of this muscle cell population.

### *Gata3 is necessary for the formation of the pyloric OLM fascicle*

Since cells of the dorsal pyloric OLM express both *Gata3* and *Nkx2-5* (Figure 2.5), we next examined the consequences of germline *Gata3* loss on the development of this fascicle. Though germline *Gata3* deficiency results in early embryonic lethality, *Gata3* null embryos can be pharmacologically rescued by catecholamine administration *in utero* and will survive until birth, permitting the analysis of *Gata3* function in later fetal development[110, 113]. We found no obvious pyloric abnormalities in *Gata3* null animals at E14.5 (data not shown). However, by E16.5, *Gata3* deficiency results in nearly complete absence of the OLM fascicle (Figure 2.10Aiv-vi, asterisks), a phenotype highly similar to that seen after *Nkx2-5* conditional deletion.

### *Loss of Gata3 or Nkx2-5 alters ICM morphogenesis and pyloric sphincter constriction*

As shown in Figures 4A and 5A, conditional *Nkx2-5* deletion or germline *Gata3* deficiency leads to loss of the dorsal OLM fascicle. In both cases, at E16.5, the shape of the dorsal ICM is clearly altered, as depicted in the tracings shown in Figure 2.10B. Since *Gata3* is not widely expressed in the ICM (Figure 2.5), the shape change detected in this mutant model must be secondary to the loss of the OLM. In addition, at E18.5, the pyloric sphincter constriction is attenuated in both genetic deficiency models; compared to wild type (95% CI = 231-266 μm), the constriction is

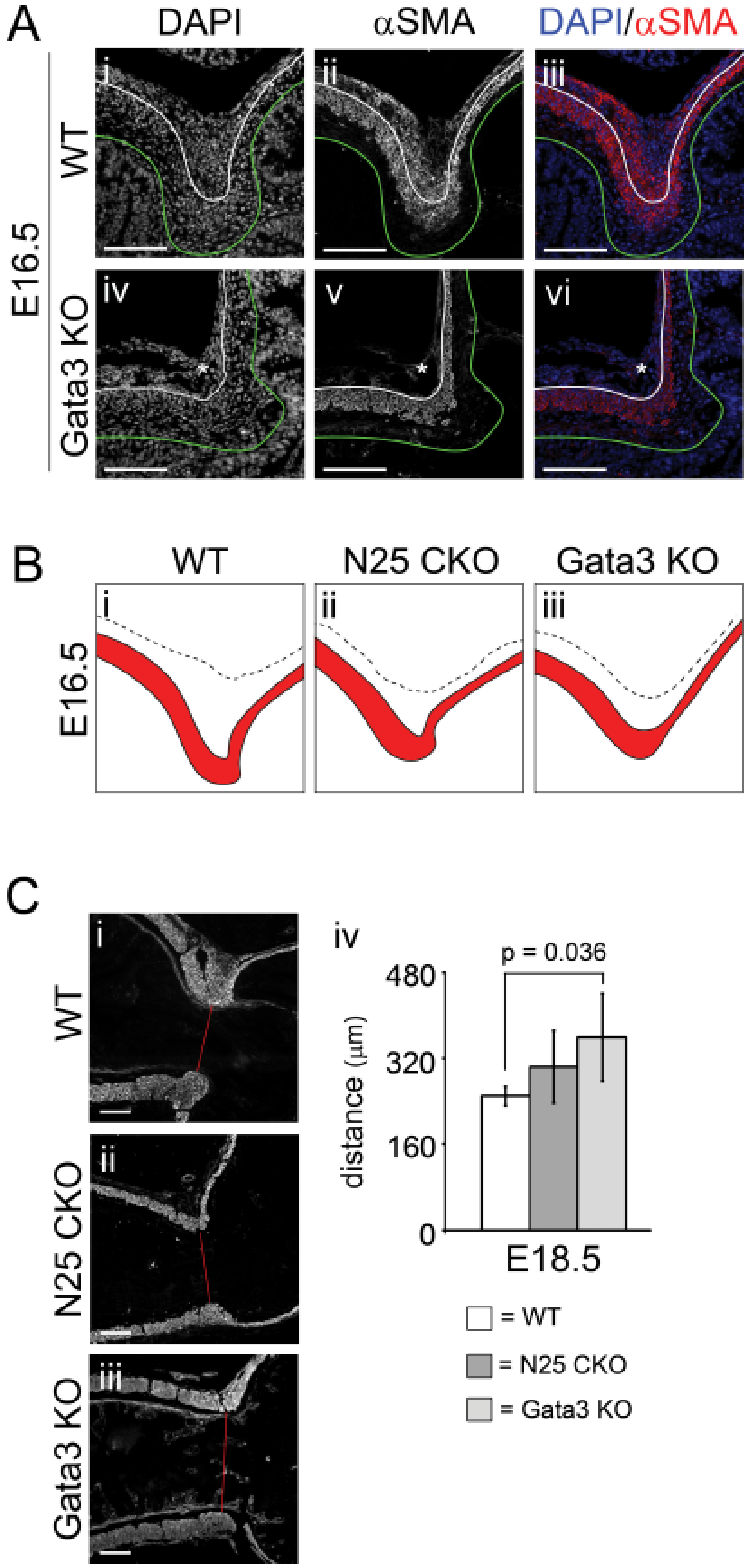

A
DAPI
αSMA
DAPI/αSMA
E16.5
WT
Gata3 KO
i
ii
iii
iv
v
vi
*
B
WT
N25 CKO
Gata3 KO
E16.5
i
ii
iii
C
WT
N25 CKO
Gata3 KO
i
ii
iii
iv
p = 0.036
distance (μm)
480
320
160
0
E18.5
= WT
= N25 CKO
= Gata3 KO

*Figure 2.10 Gata3 is required for formation of the dorsal pyloric OLM; absence of Gata3 or loss of Nkx2-5 alters ICM shape and pyloric sphincter constriction.*
(A) Immunofluorescence of (i-iii) WT or (iv-vi) Gata3lacZ/lacZ (Gata3 KO) pylorus at E16.5: (i,iv) DAPI; (ii,v) αSMA; or (iii,vi) merged. Germline deficiency of Gata3 results in nearly complete absence of αSMA positive cells in the dorsal pyloric OLM. (B) Tracings of the pyloric ICM (red) and OLM (white area defined by the dotted line) in WT, N25 CKO, and Gata3 KO mice at E16.5. Subtle but reproducible changes occur in the shape of the ICM in N25 CKO and Gata3 KO mice. (C) αSMA immunofluorescence of (i) WT, (ii) N25 CKO, or (iii) Gata3 KO pylorus at E18.5. Compared to WT, the pyloric sphincter constriction is somewhat reduced (wider) in N25 CKO animals and significantly attenuated in Gata3 KO (sphincter constriction measurements are shown in Civ). Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. Red lines in (Ci-iii) denote width of pyloric sphincter constriction. Asterisk = dorsal pyloric OLM. Scale bars = 100 µm. Error bars represent one standard deviation from the mean.

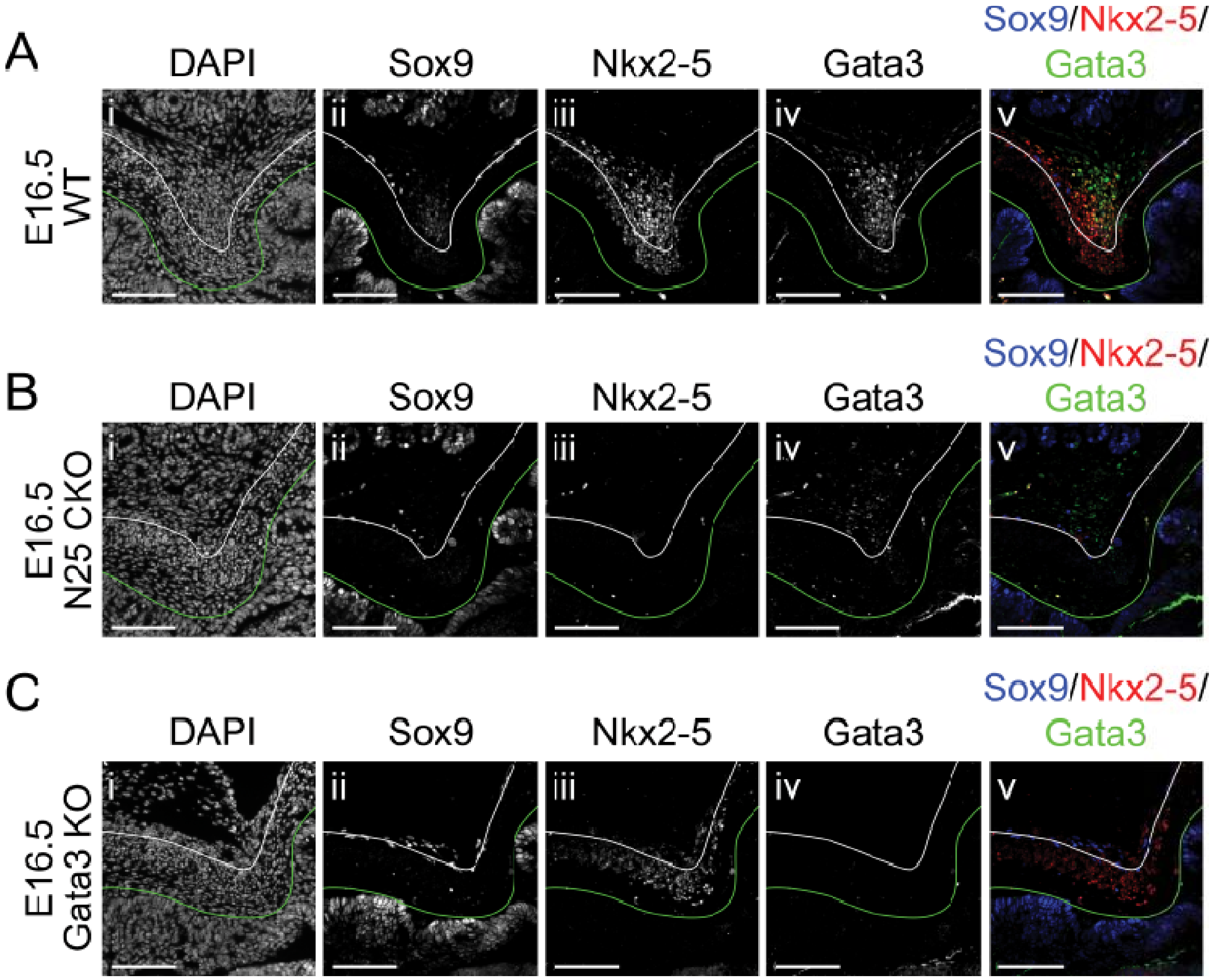

A
E16.5
WT
DAPI
Sox9
Nkx2-5
Gata3
Sox9/Nkx2-5/
Gata3
i
ii
iii
iv
v
B
E16.5
N25 CKO
DAPI
Sox9
Nkx2-5
Gata3
Sox9/Nkx2-5/
Gata3
i
ii
iii
iv
v
C
E16.5
Gata3 KO
DAPI
Sox9
Nkx2-5
Gata3
Sox9/Nkx2-5/
Gata3
i
ii
iii
iv
v

*Figure 2.11 Regulatory hierarchy among Nkx2-5, Gata3 and Sox9 in the dorsal pyloric OLM.* Immunofluorescence of (A) WT, (B) N25 CKO, or (C) Gata3 KO pylorus at E16.5: (i) DAPI; (ii) SOX9; (iii) NKX2-5; (iv) GATA3; or (v) merged. (A-C) Conditional loss of Nkx2-5 effectively eliminates dorsal OLM NKX2-5 expression, with concomitant loss of SOX9 expression, but does not abrogate GATA3 expression. Germline deficiency of Gata3 is associated with the complete absence of dorsal OLM SOX9 expression, but NKX2-5 is expressed in rare remaining dorsal OLM cells. Stomach is left; duodenum is right; and, dorsal is

40% wider in *Gata3* null animals (95% CI = 267-450 µm, $P < 0.05$) and 22% wider after conditional *Nkx2-5* deletion (95% CI = 228-379 µm, $P = 0.13$) (Figure 2.10C).

### *A regulatory hierarchy of pyloric transcription factors*

We next examined the expression of NKX2-5, GATA3 and SOX9 in E16.5 pyloric smooth muscle after conditional Nkx2-5 deletion or in Gata3 null mice. Deletion of Nkx2-5 starting at E14.5 effectively ablates NKX2-5 expression in the majority of cells at the E16.5 pylorus (Figure 2.11Biii). While SOX9 staining is absent in smooth muscle cells (Figure 2.11Bii), GATA3 expression persists in NKX2-5 negative cells of the OLM remnant (Figure 2.11Biv), suggesting that *Nkx2-5* is required for *Sox9* but not *Gata3* expression in this domain.

In *Gata3* null mice, GATA3 expression is absent (Figure 2.11Civ), as is SOX9 in smooth muscle cells (Figure 2.11Cii), but NKX2-5 expression is readily apparent in cells of the remaining OLM territory (Figure 2.11Ciii). Thus, *Gata3* is required for *Sox9* but not *Nkx2-5* expression in the dorsal pyloric OLM. Taken together, these data reveal a pyloric transcriptional hierarchy in which *Nkx2-5* and *Gata3* are independently required to drive *Sox9* expression and, consequently, smooth muscle development within the dorsal OLM fascicle.

### *Loss of Nkx2-5 or Gata3 does not affect the epithelial pyloric border*

In the chick, perturbation of mesenchymal NKX2-5 activity changes the character of the pyloric epithelium, suggesting that signaling molecules driven by NKX2-5 are responsible for dictating epithelial phenotype[5, 78]. In the mouse, the distinct characters of the stomach and duodenal epithelia are established between E14.5 and E16.5[6, 7], a time coincident with the development of the OLM fascicle (Figure 2.2). Therefore, we assessed the integrity of the epithelial stomach-

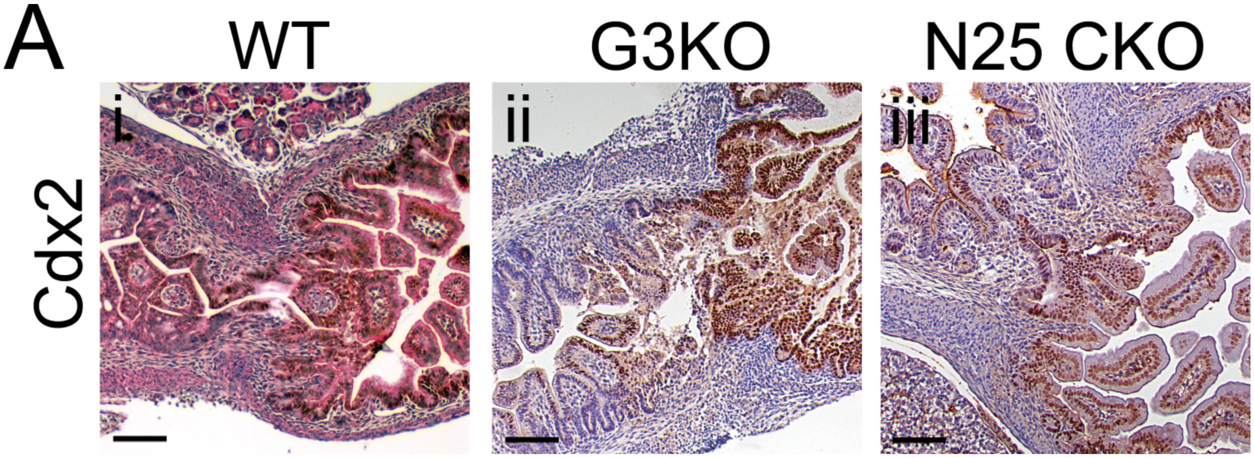


*Figure 2.12 The epithelial pyloric border is unaltered in the absence of Nkx2-5 or Gata3.* CDX2 immunohistochemistry in (A) WT, (B) Gata3lacZ/lacZ (G3 KO), or (C) N25 CKO pylorus at E18.5. (A) CDX2 is expressed in intestinal but not antral epithelial cells (i.e., the epithelial pyloric border) in WT pylorus. (B-C) Despite concomitant pyloric muscular hypoplasia, this boundary of epithelial CDX2 expression is preserved in N25 CKO and G3 KO mice.

intestinal (pyloric) border at E18.5 after conditional *Nkx2-5* deletion and in *Gata3* null animals by examining the expression of CDX2, an intestine-specific epithelial marker. In both models, the position of the epithelial pyloric border was similar to wild type (Figure 2.12). Thus, in the mouse, neither *Gata3* nor *Nkx2-5* is required for positioning of the epithelial pyloric border.

## 2.4 Discussion

Here, we provide a detailed analysis of normal pyloric muscle development at the cellular level, revealing an unexpectedly complex expression pattern of three critical transcription factors in these muscles. We provide evidence for a redundant regulatory mechanism that controls the development of a specific dorsal OLM fascicle at the pylorus. This fascicle is first detectable as a population of loosely organized, weakly αSMA positive smooth muscle cells at E14.5; it organizes considerably over the next two days, becoming highly αSMA positive and displacing the ICM to generate a distinct pyloric sphincter constriction by E16.5. Cells within this dorsal OLM fascicle express NKX2-5, GATA3, and SOX9. Deletion of either Nkx2-5 or Gata3 abrogates development of this OLM fascicle, results in loss of SOX9 expression, and alters pyloric muscular shape. While both Nkx2-5 and Gata3 are also expressed in other cells at the pylorus, loss of either factor appears to affect only the phenotype of triple positive cells (NKX2-5, GATA3, and SOX9) within the dorsal OLM fascicle.

This is the first study to characterize Gata3 pyloric expression at the cellular level and to establish a role for Gata3 in pyloric development. We find that GATA3 is expressed in cells on the serosal surface (Figure 2.6) and in cells that intermix with pancreatic mesenchyme (Figure

2.5); neither of these cell types co-expresses NKX2-5 or SOX9, and their functional role remains undetermined since no obvious phenotype was detected in serosa or pancreas in *Gata3* deficient mice. In contrast, the effect of *Gata3* loss on the pyloric OLM fascicle is dramatic and directly mirrors the effects of *Nkx2-5* loss. Despite this similarity in phenotype, we found no clear evidence of a transcriptional relationship between *Gata3* and *Nkx2-5*. Further supindependent regulation of these factors, conditional overexpression of *Wnt9b* or activation of a stabilized form of beta-catenin in the distal stomach and pylorus affects *Nkx2-5* and *Gata3* expression oppositely: the *Nkx2-5* domain is expanded, while expression of *Gata3* is abolished[109].

Interestingly, both *Nkx2-5* and *Gata3* are required for pyloric sphincter morphogenesis and both directly or indirectly regulate *Sox9* expression. Three other transcription factors, *Barx1*, *Bapx1*, and *Six2*, are also important for pyloric sphincter development and pyloric *Sox9* expression[80, 105, 107, 108]. All three factors are expressed in distal stomach as well as pylorus. It has been proposed that *Bapx1* functions downstream of *Barx1*, since its expression is lost in *Barx1* mutant mice[105]. Interestingly, loss of *Bapx1* does not affect *Nkx2-5* expression, but gene expression microarrays demonstrate decreased *Sox9* in *Bapx1* null animals[105, 108]. Thus, *Bapx1* may regulate *Sox9* expression directly or indirectly via *Gata3*. Loss of *Six2* also results in attenuation of the pyloric sphincter constriction and is associated with transient loss of *Nkx2-5* and complete loss of SOX9 expression[80]. Thus, all of these models converge on *Sox9* and given its expression in the OLM (Figure 2.5), it will be important to directly determine whether *Sox9* is required for development of this OLM fascicle and establishment of pyloric sphincter constriction. Testing this possibility will require a transgenic *Cre* driver that is active in the pyloric mesenchyme but not the epithelium, where *Sox9* plays a critical role in establishing the stem cell zone[126, 127].

Both *Nkx2-5* and *Gata3* are important for proper differentiation of the OLM fascicle, since in their absence, cells are disorganized and only weakly αSMA positive. Even very late deletion of *Nkx2-5* (after the OLM has fully developed) results in an OLM fascicle that is reduced in size, organization, and differentiation. Co-regulation of muscle differentiation genes by NKX and GATA family members has been seen in other systems: NKX3-2 and GATA6 cooperatively activate smooth muscle genes (e.g., *Itga1*, *SM22α*, and *Cald1*), while NKX2-5 and GATA4 co-activate cardiac muscle genes (e.g., *Anf*)[128]. In both cases, co-regulation also involves serum response factor (SRF), an important regulator of muscle gene expression in differentiating cardiac and smooth muscle. These three proteins together (SRF/GATA/NKX) synergistically activate target gene expression at levels 5-10 fold greater than SRF/GATA or SRF/NKX alone[128]. Thus, it is possible that loss of either *Nkx2-5* or *Gata3* disrupts ternary complexes of GATA3, NKX2-5, and SRF that are required for the expression of genes involved in the differentiation and/or maintenance of pyloric smooth muscle. This remains to be directly tested.

Failure to establish or maintain the proper pyloric musculature, as seen in both the conditional *Nkx2-5* deletion and *Gata3* null models examined here, could potentially underlie some cases of primary duodenogastric reflux, a rare and poorly understood condition involving excessive reflux of bile acids from the duodenum to the stomach[19]. IHPS, in contrast, presents as pyloric obstruction as opposed to lack of constriction. Given that recent GWAS data link nucleotide polymorphisms near the *NKX2-5* gene locus to IHPS[13], our results would predict that some forms of IHPS may result from overexpression of *NKX2-5* at the pylorus. For example, a change in an intergenic regulatory element could readily account for increased pyloric *NKX2-5* expression. Indeed, an upstream enhancer that drives pyloric *Nkx2-5* expression in mice has already been described[124] and it will be interesting to examine the sequence of this region in patients with IHPS. Additionally, our data suggest that the search for linked polymorphisms in IHPS should be expanded to include *GATA3* and, potentially, *SOX9*.

## Acknowledgements

The work presented in this chapter contains contributions from Brianna Sabol. In addition, it would not have been possible without the University of Michigan Microscopy and Imaging Laboratory or the University Laboratory Animal Medicine department. My co-first author on this work is Aaron Udager, who performed many of the preliminary experiments and provided invaluable guidance and feedback. The other co-authors on this work are David A. Saenz, Martina Schinke, Takashi Moriguchi, Patrick Y. Jay, Kim-Chew Lim, James Douglas Engel, and Deborah L. Gumucio.

*Table 1. Gata3 KO 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)
*P* = 0.132 (N25 CKO vs. WT)

*Table 2. N25 CKO 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* = 0.023 (N25 CKO vs. WT)

| | 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)

*Table 3. N25 CKO 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)
$P = 0.002$ (Nkx2-5 #; N25 CKO vs. WT)
$P = 0.661$ (Casp3 #; N25 CKO vs. WT)
$P = 0.010$ (Nkx2-5 %; N25 CKO vs. WT)
$P = 0.066$ (Casp3 %; N25 CKO vs. WT)

# Chapter 3 [2]

# Development of the Pyloric Ligaments

## Summary

A number of ligament structures connect the stomach and intestine with surrounding organs, most of which consist primarily of vascular and nervous tissue that provides perfusion and nerve connectivity between the connected organs. On the lesser curvature side of the stomach, a pair of superficial cord-like structures, referred to previously as gastric ligaments, extends from the pyloric constriction to the gastroesophageal junction. Though these structures have been recognized in many mammalian species, very little is known about their cellular composition, function or development. We examined cellular structure and gene expression (of *αSMA*, peripherin, CD31) in these organs at E12.5, E14.5, E16.5, P0 and P30. We show that the gastric ligaments originate at E12.5 as *αSMA* positive outgrowths from the pyloric outer longitudinal muscle (OLM) and extend to the esophagus around birth. These ligaments are composed of smooth muscle and covered by serosa. Throughout their development, the gastric ligaments express the transcription factors *Gata3* and *Nkx2-5*. Since both of these factors are also expressed in the pyloric OLM and necessary for its proper development (Udager et al. unpublished data), we examined the pyloric gastric ligaments for expression of these transcription factors and assessed ligament integrity in mouse models that lack each gene. As the ligaments form, the expression domain of both factors expands along the growing ligament structures. Germline loss

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[2] This chapter is being prepared for submission to Developmental Dynamics. It includes contributions from Aaron Udager, David Saenz, and Deborah Gumucio

of *Gata3* impairs development of the gastric ligaments. Conditional loss of *Nkx2-5*, at a time after ligaments have formed, results in reduced *αSMA* expression and hence reduced differentiation of their smooth muscle component. In light of their direct structural and molecular connection to the pyloric OLM, we propose to rename these structures as: *pyloric ligaments*. We speculate that they may act, together with the OLM, to flex or stabilize the pyloric region, actions that may promote proper pyloric sphincter function.

### 3.1 Introduction

The connective tissue ligaments of the gastrointestinal tract perform a critical role in supporting gastrointestinal motility and perfusion. These ligaments are named for their points of origin and insertion and include the gastrosplenic, gastrophrenic, gastrocolic, hepatogastric, hepatoduodenal, and splenorenal ligaments, the suspensory ligament of the duodenum (ligament of Treitz) and the gastric ligaments. Of these, the ligament of Treitz and the gastric ligaments are the only ones that do not consist primarily of neurovascular tissue and have been speculated to be similar in function [125].

The ligament of Treitz is thought to provide structural support within the gastrointestinal tract. Morphologically, it is sometimes described as consisting of two distinct subunits [129]. The upper part, also called the Hilfsmuskel, is a skeletal muscle structure that extends from the diaphragmatic crus to the celiac axis; the lower part, known as the suspensory muscle of the duodenum, is composed of smooth muscle and extends from the duodenum upward to the celiac axis [129, 130]. However, these two segments are compositionally different and appear to have distinct origins, with the Hilfsmuskel originating from the diaphragm [131] and the suspensory muscle of the duodenum originating form the outer longitudinal smooth muscle of the duodenum [131, 132]. Therefore, it has been argued that these two structures are distinct entities [130, 133]. Functionally, the ligament of Treitz/suspensory muscle of the duodenum is thought to maintain the flexure of the duodenojejunal junction and facilitate the embryologic rotation of the bowel [130, 134].

The gastric ligaments consist of a pair of bilateral, superficial cord-like structures, located on the lesser curvature of the stomach, that extend from the pylorus to the connective tissue surrounding the esophagus [125]. The development and composition of these ligaments have not been carefully examined, but they have been speculated to control the angle of flexure between the duodenum and antrum [125]. Recently, while characterizing gene expression pattern in the inner circular (ICM) and outer smooth longitudinal (OLM) smooth muscle layers of the pyloric sphincter (Udager et. al. manuscript submitted), we discovered that expression domains for the transcription factors *Nkx2-5* and *Gata3* appeared to be physically contiguous between the gastric ligaments and the pyloric OLM (Udager et al. Manuscript submitted). After germline deletion of *Gata3* or conditional ablation of *Nkx2-5* from developing embryos, the pyloric OLM is sharply attenuated and the remaining cells are only weakly αSMA positive. Thus, both *Gata3* and *Nkx2-5* are required for proper development of the pyloric OLM. However the fate of the gastric ligaments was not assessed in that study.

Here, we examine the development of the gastric ligaments between E12.5 and P30. We demonstrate that these structures contain minimal neurovascular tissue, but are composed primarily of smooth muscle and lined by serosa. As was the case for the OLM, both *Gata3* and

*Nkx2-5* are required for proper maturation of the gastric ligaments. Also similar to the pyloric OLM, *Sox9* is expressed in the gastric ligaments and loss of either *Gata3* or *Nkx2-5* results in loss of *Sox9* expression. These molecular similarities between the gastric ligaments and the OLM, combined with the physical contiguity of the OLM and ligament structures, suggests that they may work together to control the flexure of the pyloric region. On this basis, we suggest that these structures should be called "pyloric ligaments".

## 3.2 Results

### *Normal development of the pyloric ligaments*

We examined the ventral side (lesser curvature) of the pyloric region between E14.5 and E16.5 in wild-type mice in order to assess the origin of the pyloric ligaments and characterize their development. The pyloric ligaments appear as outgrowths of the pyloric OLM at E14.5 (Figure 3.1). At this time, the ligaments extend anteriorly along the lesser curvature of the stomach and do not thin significantly from their base to their tip. By E16.5, the ligaments extend further up the lesser curvature of the stomach. They appear thinner at this point and terminate in connective tissue around the base of the esophagus. By E16.5, the cells of the ligament core are elongated in the anterior/posterior direction and the lining cells have further flattened (Figure 3.1).

### *Tissue characterization of the developing pyloric ligaments*

To characterize the cell populations present within the pyloric ligaments, we stained sectioned tissue for αSMA, peripherin, and CD31 for muscle, nervous, and vascular tissue respectively. At early timepoints, while the ligament is a short outgrowth of the pyloric tissue (Figure 3.2A), none

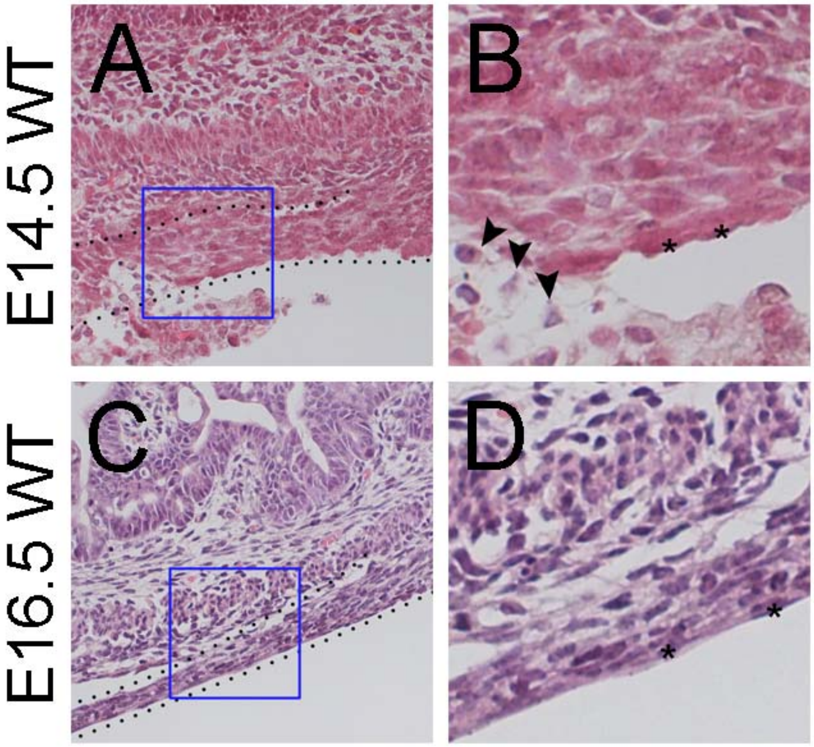

*Figure 3.1 Development of the pyloric ligament*
H&E stained sectioned tissue of E14.5 (A-B) and E16.5 (C-D) pyloric tissue. (A) The early pyloric ligament (indicated by the dotted black line) is an outgrowth of the ventral side of the pylorus. (B) The ligament is lined by a unique layer of narrow, elongated cells (asterisks), and anchored to the esophagus and ventral stomach by loose connective tissue (arrowheads). (C) By E16.5, the ligament thins and elongates, while maintaining its overall architecture.

of these markers is expressed at significant levels (Figure 3.2A-D). Over the next two days, *αSMA* expression within these cells strengthens (Figure 3.2G, H,K,L) considerably. In contrast, periferin staining reveals no obvious nervous tissue within the gastric ligament at E14.5 (Figure 20B) and limited nervous tissue at E16.5 (Figure 3.2 J). Robust peripherin expression is detected in the cells of the myenteric plexuses within the antral wall at both E14.5 (Figure 3.2B) and E16.5 (Figure 3.2J). Similarly, limited vascular endothelial tissue is present within the pyloric ligaments (Figure 3.2F). Thus, the pyloric ligaments are primarily muscular structures.

### *Expression of pyloric transcription factors in the pyloric ligaments*

Our earlier studies of *Nkx2-5*, *Gata3* and *Sox9* revealed that all three of these factors are co-expressed in a fascicle of OLM at the pylorus (Udager et al., manuscript submitted; see Chapter 2). Additionally, whole mount analyses suggested that *Nkx2-5* and *Gata3* were expressed in the pyloric ligaments and that this expression domain extended directly from the pyloric OLM. However, the expression of these factors was not examined in the pyloric ligaments on a cellular basis in that study. Here, we show that in E14.5 sectioned tissue, expression of both *Gata3* and *Nkx2-5* is detectable in the central rounded cells of the ligaments (Figure 3.3). Additionally, the flattened serosal cells surrounding the ligaments are GATA3 positive but NKX2-5 negative, similar to some of the serosal cells seen on the outer edges of the OLM (Udager et al., Manuscript submitted; Chapter 2). SOX9 expression is also present within the pyloric ligament muscle (Figure 3.3). These expression profiles remain unchanged at E16.5 (data not shown).

### *Loss of Gata3 or Nkx2-5 affects development of the pyloric ligaments*

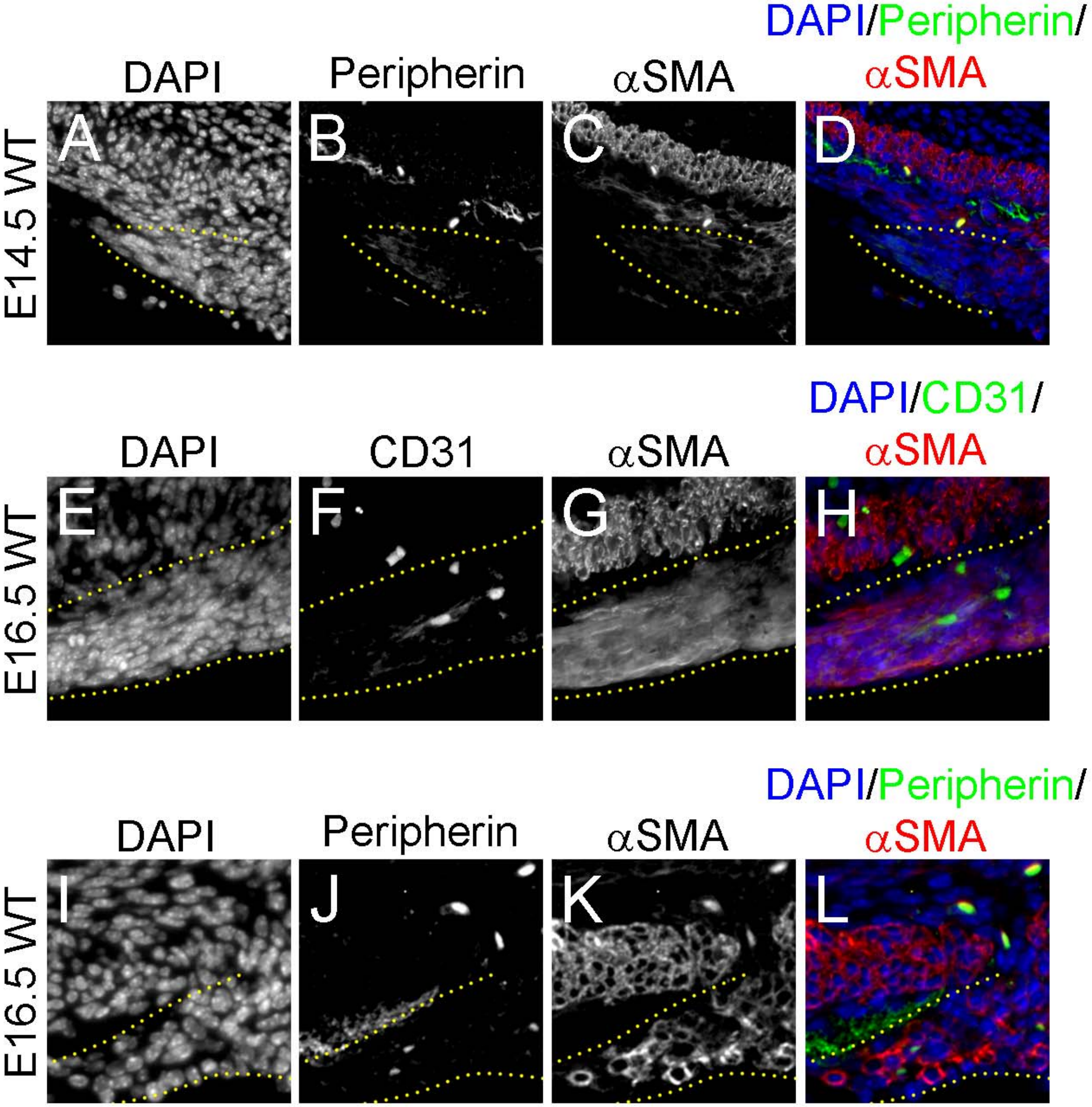

*Figure 3.2 The pyloric ligaments are muscular structures*
Frozen (E-H) or paraffin (A-D, I-L) sectioned tissue of E14.5 (A-D) and E16.5 (E-L) wild-type tissue shows the expression of muscular and neurovascular structures in and around the pyloric ligments. (A-D) At E14.5, there is limited αSMA expression within the developing pyloric cords (white arrowheads), and peripherin expression is largely limited to the enteric plexes associated with the ICM. (E-H) Limited vascular tissue is visible at E16.5, but the intensity of □SMA staining has increased. (I-L) There is limited expression of peripherin within the cells of the pyloric cord but robust αSMA staining is detectable.

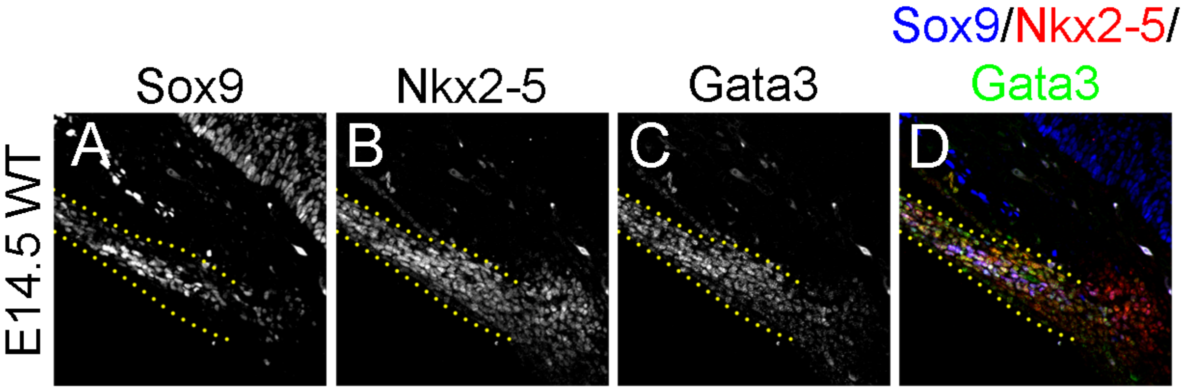

*Figure 3.3 Pyloric transcription factors are expressed in the pyloric ligaments*
Paraffin sectioned tissue from an E14.5 wild-type mouse pylorus. (A) *Sox9* is expressed within the core of the pyloric ligament at E14.5. (B) Nkx2-5 expression is strong throughout the ligaments, with expression extending from the ligament onto the ventral aspect of the pylorus. (C) *Gata3* is expressed throughout the ligament, but less robustly in the ventral pylorus. (D) An overlay of *Sox9* (blue), Nkx2-5 (red), and *Gata3* (green) expression in the pyloric ligament reveals a subset of cells in the core of the ligament in which all three transcription factors are coexpressed.

We previously demonstrated that, in the absence of either *Gata3* or *Nkx2-5*, differentiation of pyloric muscular tissue is reduced. Furthermore, we showed the loss of one of these factors did not affect the expression of the other (Udager et al., Manuscript submitted; Chapter 2). Thus, we examined the consequences of loss of *Gata3* or *Nkx2-5* on the pyloric ligaments. In *Gata3*$^{\mathrm{lacZ/lacZ}}$ knockout mice, the pyloric ligaments are severely reduced in size (Figure 3.4). The attenuated ligaments contain cells that are only weakly αSMA positive. The *Gata3* deficient vestigial ligaments still express NKX2-5, suggesting that, as in the OLM, *Gata3* is not required for the maintenance of *Nkx2-5* expression in these structures (Figure 3.4A-D).

Because germline *Nkx2-5* deletion is lethal in the early embryo due to its role in heart development, we utilized a conditional deletion model to examine the role of this factor in pyloric ligament development. *Nkx2-5*$^{\mathrm{f/f}}$ mice were mated to CAGG-Cre-ER mice, tamoxifen was given at E14.5 and E15.5 and tissues were harvested at E16.5. The pyloric ligaments (which had fully formed by this time) were similar to wild type in size and shape. However, the central cells did not express αSMA, suggesting that muscular maturation is inhibited (Figure 3.4E-H). While no NKX2-5 staining was visible in the ligament (confirming the knockout), GATA3 was expressed (Figure 3.4G). Thus, no epistatic relationship can be demonstrated between *Gata3* and *Nkx2-5* in the OLM (Udager et al., Manuscript submitted; Chapter 2) or in the pyloric ligaments.

### *Three dimensional reconstruction reveals continuity between the pyloric ligaments and the pyloric OLM*

In earlier studies (Udager et al., Manuscript submitted; Chapter 2), we noted that the expression of *Nkx2-5* and *Gata3* at the pylorus appears to be continuous with expression in the gastric

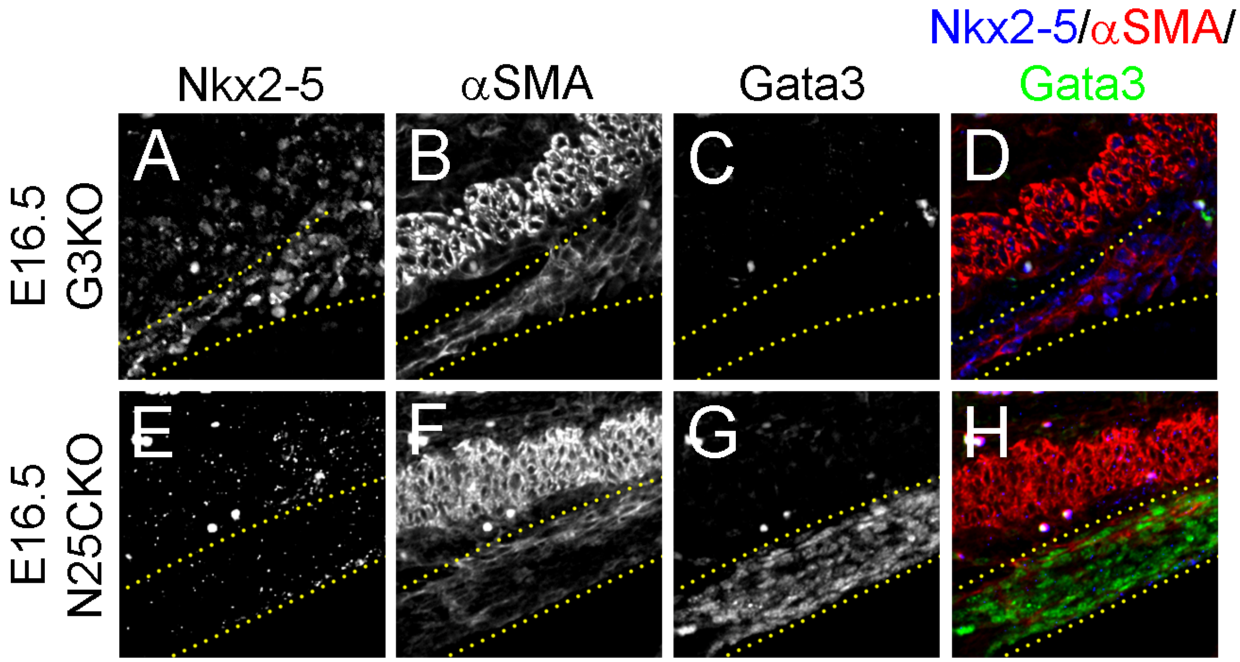

*Figure 3.4 Loss of Nkx2-5 or Gata3 leads to decreased smooth muscle expression*
Sectioned tissue from E16.5 *Gata3*lacZ/lacZ (G3KO) or Nkx2-5f/f;Cagg-CreERT2 (N25CKO) mice. (A) Germline loss of *Gata3* does not abrogate Nkx2-5 expression in the developing pyloric cord. (B) The cord itself is thinner and expresses less αSMA than wild-type. (C) *Gata3* expression is absent, confirming the knockout. (D) An overlay of Nkx2-5(blue), αSMA (red), and *Gata3* (Green) shows that *Gata3* expression is absent while Nkx2-5 expression is still robust within the cord. (E) In Nkx2-5 deletion mice Nkx2-5 expression is undetectable. (F) While the cord thickness is similar to wild type, there is a significant decrease in αSMA expression within it. (G) *Gata3* expression is still robust in the pyloric cord after the deletion of Nkx2-5. (H) An overlay of all three genes.

ligaments. We also noted a subtle difference in the expression patterns of these two factors, in that expression of NKX2-5 appeared to be fully circumferential while that of GATA3 exhibited a gap on the ventral side of the pylorus, between the two pyloric ligaments. To better understand the relationship between the pyloric OLM and the ligaments, we used serial cross-sections and Imaris software to rebuild a portion of the pylorus in three dimensions. Sections were stained for αSMA and GATA3 and the continuity of staining was assessed in both individual sections and in the rebuilt three-dimensional image. As shown in Figure 3.5, we found that the muscle of the pyloric OLM is indeed continuous with that of the pyloric ligaments. Additionally, continuity of expression of GATA3 is demonstrable. Moreover, on the ventral side of the pylorus, αSMA staining reveals that the OLM layer (which expresses both *Nkx2-5* and *Gata3*, see Chapter 2) is extremely thin or missing altogether, while the ICM layer (which expresses *Nkx2-5*, but not *Gata3*, see Chapter 2) is fully circumferential. These reconstructions provide an explanation for the apparent gap in *Gata3* expression on the ventral side of the pylorus seen in whole mount sections.

## 3.3 Discussion

This analysis of the development of the pyloric ligaments reveals that these ligaments extend from their pyloric origin at E14.5, with expression extending to the esophagus by E16.5. Three dimensional reconstructions reveal clear continuity between the pyloric ligaments and the outer longitudinal muscle of the pylorus. Additionally, these reconstructions confirm the lack of OLM on the ventral side of the stomach in the region between the pyloric ligaments, noted earlier (Udager et al., manuscript submitted). Thus, the structure of this muscular fascicle resembles a saddle, with the stirrups angled anteriorly toward the esophagus (Figure 3.5). Interestingly, the

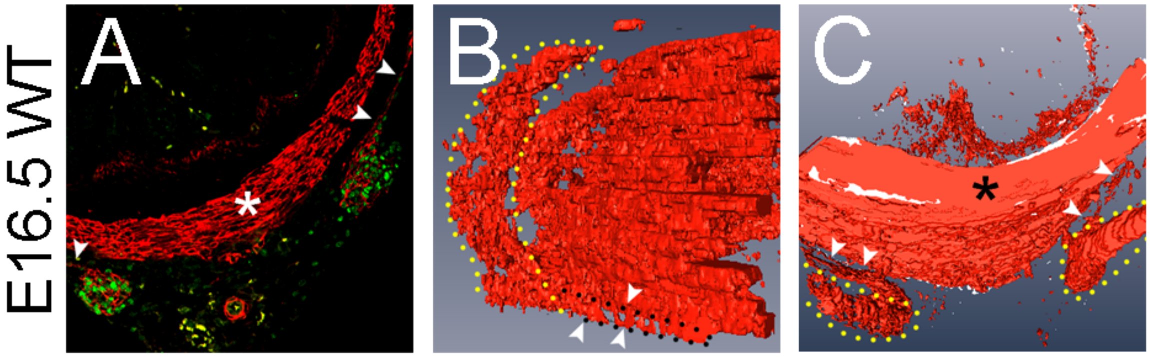

*Figure 3.5 The pyloric ligaments are continuous with the pyloric OLM*
3D reconstruction of αSMA (red) and *Gata3* (green) expression shows the pyloric ligaments are continuous with the pyloric OLM (white arrows), and are distinct from the ICM (star). (A) An anterior image of the pyloric ligaments show the ligaments descending from the ventral antrum to the level of the pylorus and joining with the pyloric OLM (white arrows). (B) This structure is better revealed with a lateral view. (C) In sections, it becomes obvious that *Gata3* expression is limited to the ligaments and the pyloric OLM (white arrows) and not the pyloric ICM.

lack of outer longitudinal muscle at the inner curvature of the pylorus is a feature that is shared by the pig stomach [1] several other species also show thin OLM in this region [1].The similarity between the pyloric ligaments and the OLM extends to the characteristic molecular expression domains that characterize these structures, as well as to the requirement for *Nkx2-5* and *Gata3*. The pyloric ligaments express *Nkx2-5*, *Gata3* and *Sox9*, as does the OLM (Udager et al, manuscript submitted). The ICM expresses *Nkx2-5*, but not the other two factors. In the absence of *Gata3*, or after the conditional deletion of *Nkx2-5*, the pyloric ligaments become attenuated and *αSMA* expression is reduced. A similar phenotype was seen in the OLM (Udager et al, manuscript submitted). Also as seen in the OLM, no dependent regulatory relationship can be discerned between *Gata3* and *Nkx2-5* (Udager et al, manuscript submitted).

These data represent the first molecular and cellular characterization of the pyloric ligaments. As muscular structures, the pyloric ligaments differ from other ligaments of the stomach, which are critical in providing neurovascular connections between the tissues [134]. Based on their position and contiguity with the OLM, connecting the ventral pylorus to the distal esophagus, it is possible that these ligaments are necessary for determining the angle between the stomach and duodenum, in a manner similar to the proposed function of the ligament of Treitz. Indeed, a role for these ligaments in flexure was suggested by Torgersen, based on anatomical and radiological analysis of pyloric regions from multiple species, including dog, pig, cat, horse and human [125]. It will be of great interest to further probe the contribution of the pyloric ligaments to pyloric function and to analyze their character in the context of idiopathic hypertrophic pyloric stenosis.

## Acknowledgements

The work in this chapter within this chapter is currently under preparation for submission. It contains contributions from Dimeng Chue. My co-first author on this work is Aaron Udager, who performed several of the preliminary experiments and provided scientific guidance and feedback. The other authors on this work are David Saenz, and Deborah L. Gumucio.

# Chapter 4

# Conclusion

## Summary

The work presented in the previous chapters expands our current understanding of pyloric development by identifying the function of *Nkx2-5* and *Gata3* at the pylorus, and relating both genes to the existing pyloric signaling network at the pylorus. We showed that both *Gata3* and *Nkx2-5* are involved in pyloric muscular development (see Chapter 2). Specifically, both *Nkx2-5* and *Gata3* are necessary for the formation of the pyloric OLM, the absence of which causes significant changes to pyloric morphology and a loss of pyloric constriction (Figure 2.10). In addition, we investigated the mechanism by which loss of *Nkx2-5* affects pyloric muscular development, showing that changes to both proliferation and apoptosis contribute to the loss of the pyloric OLM. Unlike the avian model, loss of *Nkx2-5* did not affect the character of the pyloric epithelium, suggesting that its function at the mammalian pylorus is distinct from that in the avian pylorus. Our data also extend the current understanding of *Sox9* expression at the pylorus. As with *Nkx2-5*, avian studies of *Sox9* demonstrate that it is necessary for the proper formation of the pyloric epithelium. Mammalian studies have also shown that *Sox9* expression is affected by loss of antral *Six2*. Our findings extend these observations by showing that pyloric *Sox9* is expressed in a subset of *Nkx2-5* and *Gata3* double positive cells. In addition, loss of either *Nkx2-5* or *Gata3* abrogates *Sox9* expression, suggesting that *Sox9* expression depends on both of these genes. We also demonstrated that *Nkx2-5*, *Gata3*, and *Sox9* are expressed within the newly characterized pyloric cords. Earlier studies had described these structures as gastric ligaments, but their tissue composition and function was not examined [125].Our data demonstrate that these are in fact muscular structures whose formation depends on the expression of *Nkx2-5* and *Gata3*. These findings present a series of new questions. What is the relationship (if any) between *Nkx2-5* and IHPS? If *Nkx2-5* and *Gata3* are expressed independently of one another, what accounts for their similar expression domains and similar functions in pyloric muscular development? Does loss of pyloric *Sox9* affect pyloric muscular development? What is the precise function of the pyloric cords, and how do they perform this function? In this chapter, I will address these questions and propose experiments that will allow us to answer them. Then I will discuss the implications of these answers and the eventual direction of pyloric developmental research.

## 4.1 Investigating the relationship between *Nkx2-5* and IHPS

Among the many implications of the work presented here, the most clinically important is that *Nkx2-5* is demonstrably involved in pyloric muscular development (see Chapter 2). Since *Nkx2-5* has been implicated in the pathogenesis of IHPS in recent GWAS studies [13], our findings provide a hypothesis for the mechanism by which *Nkx2-5* gives rise to IHPS: specifically, that IHPS might result from overexpression of *Nkx2-5* shortly after birth.

The most direct assay for this hypothesis would be to generate a mouse model that overexpresses NKX2-5. I would utilize a previously described *Nkx2-5* enhancer that has a pyloric-specific expression domain as a driver for *Nkx2-5* cDNA expression [77]. Additionally, since wild-type NKX2-5 protein shows relatively low levels of transcriptional activity, due to the presence of a negative transactivating domain at the C-terminus of the protein [135], I will employ a highly active version of NKX2-5 with residues 203-318 deleted (NKX2-5ΔC). This construct would then be used to create a transgenic mouse that would be assayed for morphological and signaling changes at the pylorus. Examining whole mount samples from such a mouse would presumably show ringed muscular expansion at the pylorus. In sections, we would expect this to be reflected by an expansion of the pyloric OLM, since loss of NKX2-5 appears to exclusively affect this tissue layer, though changes in the ICM might also be seen. These findings would demonstrate a direct link between NKX2-5 expression and pyloric OLM development. If such a muscular expansion were absent in the *Nkx2-5* overexpression model, it would suggest that *Nkx2-5* is necessary for the formation of the pyloric OLM, but it is not sufficient for its proliferation.

Given the conflicting nature of the existing data on pyloric signaling [78, 80, 108, 109], and the lack of data demonstrating direct relationships between pyloric signaling molecules and transcription factors, it is difficult to speculate on how supraphysiologic NKX2-5 expression would affect the signaling environment at the pylorus. Since both our data and earlier reports have demonstrated that pyloric NKX2-5 and SOX9 are modulated in concert by different signaling and transcription factors, it may be that SOX9 expression would expand after NKX2-5 overexpression. Also, since our data appear to suggest that *Gata3* is expressed within the differentiated pyloric OLM it seems reasonable to hypothesize that *Gata3* expression there would also increase. Presumably, however, this would be passive, due to expansion of the muscle rather than via direct regulation of *Gata3* by *Nkx2-5*.

Given the GWAS results, it would be of great interest to secure specimens from IHPS patients and to determine the expression levels of NKX2-5 (as well as GATA3 and SOX9) within those samples relative to those seen in cadaverous controls. Since we see such large discrepancies between the avian and mammalian model, it would be reassuring to see our findings recapitulated in human tissue as it would suggest that the mouse pylorus is an appropriate model system in which to study IHPS. Finally, sequencing the *Nkx2-5* locus in patients with the identified *Nkx2-5* polymorphism could reveal the direct relationship between *Nkx2-5* and IHPS. This, in combination with functional studies, would clarify whether the causative mutation within *Nkx2-5* results in its overexpression, constitutive activation, or is completely unrelated to *Nkx2-5* expression. This finding could then be used to generate more accurate IHPS mouse models. These in turn may allow us to better understand the relationship between *Nkx2-5* and *Gata3* and determine whether the latter gene also plays a role in IHPS.

## 4.2 Regulation of ***Nkx2-5, Gata3*** by known signaling molecules at the pylorus

Interestingly, the region in which the pyloric musculature forms is marked earlier in development by the lack of lack of WNT signaling (Kiefer et al., 2012) as well as the lack of BMP signaling [79, 80]. However the regulatory relationship between *Nkx2-5* and *Gata3* and both of these signaling pathways is far from clear. Below, I will examine the rather confusing lines of evidence for the role of BMP and WNT signaling in pyloric development.

### *WNT signaling and pyloric development*

Ectopic *Wnt* expression at the pylorus alters the expression of both NKX2-5 and GATA3 [109], but has opposite effects, expanding the NKX2-5 expression domain while reducing that of GATA3. Confusing the issue, these data conflict with earlier data on effects of WNT signaling on *Nkx2-5*, as *Barx1* and *Bapx1* have both been shown to modulate gastric and pyloric WNT expression but do not appear to affect the expression of *Nkx2-5* [95, 108]. Interestingly, WNT expression is already absent at the pylorus by the time *Gata3* expression is initiated at E11.5 [7, 136] and could be absent even earlier. Indeed, WNT expression is necessary for the early pattern formation that results in the formation of the foregut-midgut junction, suggesting that it could be involved in demarcating the position of a pylorus-specific *Nkx2-5* and *Gata3* domain [136]. The simplest test of this is overexpression of *Wnt* or active *β-catenin* at the pylorus. This is exactly the approach taken by Kiefer et al. [109], but as mentioned, with opposite outcomes for *Gata3* and *Nkx2-5*. It is important to note, however, that these authors only studied *Gata3* and *Nkx2-5* expression by whole mount in situ hybridization and it is altogether possible that in the critical cells of the OLM, the overexpression of WNT ligands might have suppressed both *Nkx2-5* and *Gata3*. This needs to be carefully examined. Another caveat of the studies done by Kiefer et al. is the fact that a *Six2*-Cre driver was used to overexpress WNT9B. *Six2* is expressed throughout the antrum, but whether *Six2* is actually expressed in the cells of the OLM at the pylorus has not been established, and it is not clear whether *Six2* and NKX2-5 and *Gata3* are expressed in the same cells. Thus, while the phenotype of these mice was partial loss of pyloric stricture, their Figure 6 does seem to show that residual pyloric muscle is present [109]. It is not clear, therefore, whether *Wnt* overexpression is actually felt by the cells co-expressing GATA3 and NKX2-5 in this model. Indeed, WNT ligands are known to stick to extracellular matrix and do not diffuse very far from their source. To better test the function of WNT in regulating *Nkx2-5* and *Gata3*, it would be preferable to utilize a Cre construct that drives recombinase specifically in the cells that co-express these factors. Pyloric-specific enhancers from either *Nkx2-5* or *Gata3* could be used for this purpose. Such tools are discussed below.

### *BMP signaling and pyloric development*

The effect of BMP signaling on pyloric development is equally puzzling. Early studies indicated that the pylorus is marked by a zone that is free of *Bmp4* ligand expression [79, 80]. Additionally, expression of the Bmp antagonist, *Grem1* was detected specifically at the pylorus [7]. Thus, it was speculated that the pylorus is a zone that lacks BMP signal reception. In support of this, Dr. Kate Walton in our laboratory has utilized a BMP signaling reporter mouse from Blank et al. [137, 138], that faithfully reports the reception of BMP signals and finds no evidence of BMP signal reception at the pylorus proper. Despite this, studies in the chick model indicate that both *Nkx2-5* and *Sox9* domains at the pylorus are induced by *Bmp4*. *Bmp4* ligand is thought to be derived from surrounding stomach and intestinal mesenchyme and to diffuse into the pyloric region to regulate *Nkx2-5* and *Sox9*. Indeed, overexpression of noggin affects pyloric development in the chick model [78]. A final piece of data confusing the picture is that after the loss of *Six2*, *Bmp4* is *enhanced* at the pylorus and the pyloric constriction fails to form; this is accompanied by temporary loss of *Nkx2-5* expression and loss of *Sox9* expression, suggesting that *Bmp4* suppresses rather than activates expression of these two genes [80].

To clarify these data, it will be necessary to first re-examine the BMP reporter mice to determine whether cells that express *Nkx2-5* and *Gata3* are positive or negative for BMP signaling. (It should be mentioned here that the use of the pSmad1/5/8 antibodies to stain for BMP signal transduction at the pylorus has not been successful in our hands). Second, a *Bmp4* overexpression experiment akin to the one performed above for Wnt, and using a pyloric-specific driver, would reveal whether restriction of Bmp from the pylorus is necessary for proper pyloric development and whether this affects *Nkx2-5* and *Gata3* expression. Interestingly, we have examined the *Grem1* knockout mice and find no phenotype at the pylorus, indicating that if a Bmp-free zone is required at the pylorus, such a zone is not established solely by the expression of *Grem1* (Figure 4.1, 4.2).

### *Clues from pyloric-specific enhancers*

Additional clues to factors that might be responsible for the regulation of *Nkx2-5* and *Gata3* might be found in the enhancers that drive pyloric specific expression of both of these transcription factors, discussed below [77, 139](see Section 4.4, Figure 4.3). TRANSFAC analysis of both enhancers (which identifies putative transcription factor binding sites) identified possible sites for PAX4 and PAX6 in both enhancers. However, these are thought to be epithelial transcription factors, and it is not clear that either one of them is expressed at the pylorus. It is possible that these types of sites are occupied by other transcription factors. Nevertheless, this analysis provides little insight into common upstream regulatory mechanisms for *Nkx2-5* and *Gata3*. It is possible that additional pyloric-specific enhancers exist for each gene and that these still unidentified regulatory regions will hold the clue to a common upstream regulatory pathway. Thus, while the data demonstrate that *Barx1*, *Bapx1*, *Six2*, *Nkx2-5*, and *Gata3* all affect pyloric muscular development in a similar manner, there is no current data identifying a possible “master regulator” of pyloric smooth muscle among these genes.

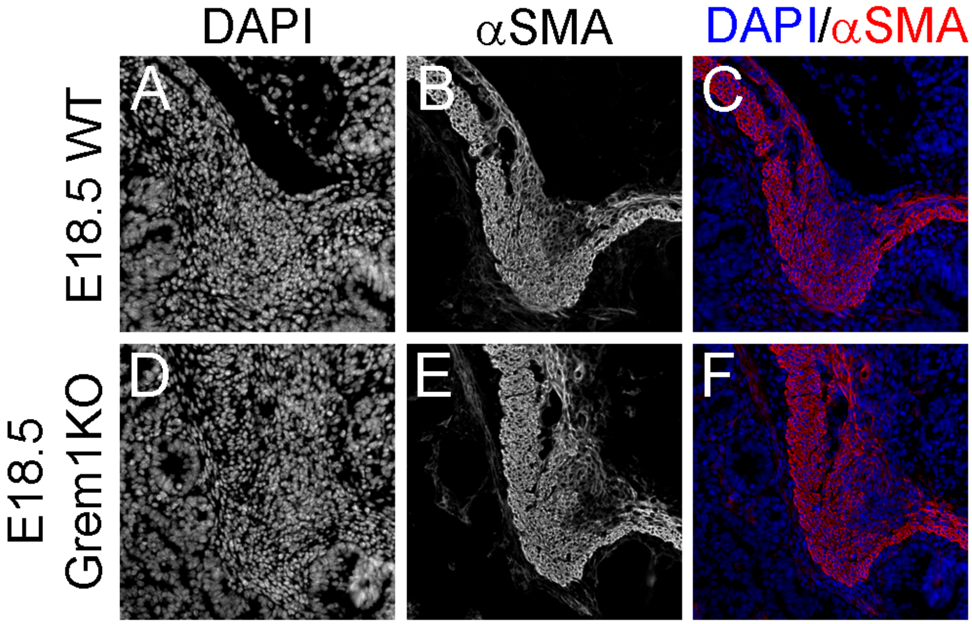

*Figure 4.1 Smooth muscle expression in the Grem1 knockout mouse*
E18.5 sections of wild-type (A-C) or Grem1 knockout (D-F) pylorus shows that smooth muscle expression is undisturbed in the absence of Grem1. Both the OLM and the ICM are of similar thickness in the knockout (E) and the wild-type (B) pylorus.

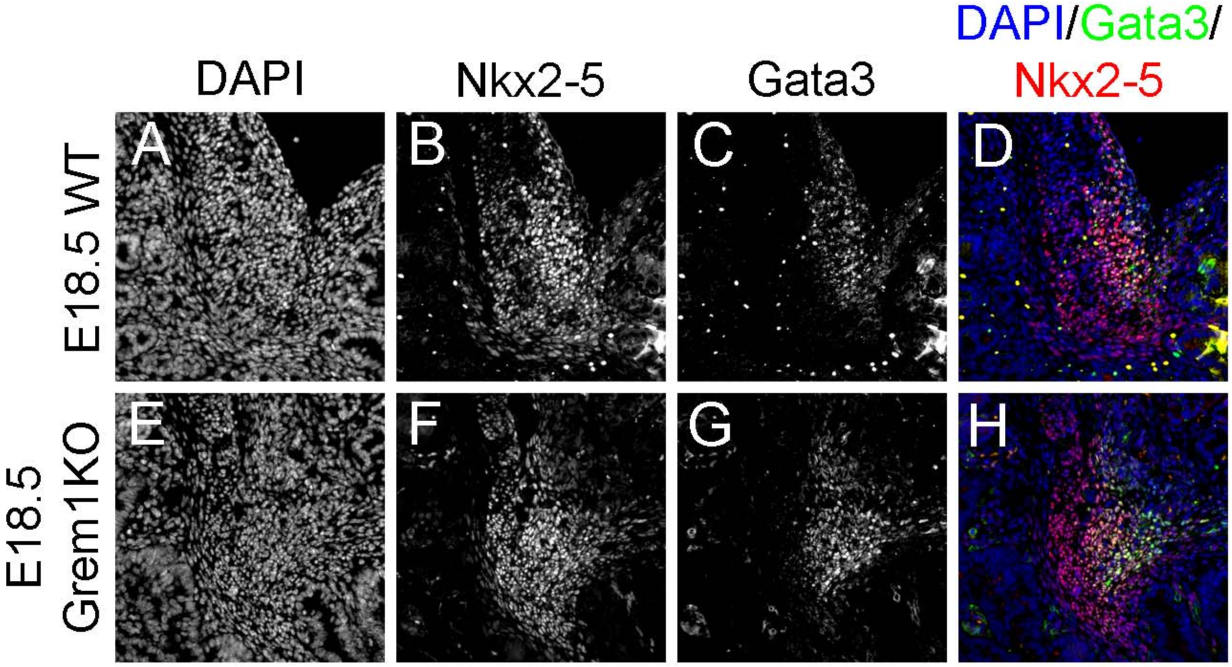

*Figure 4.2 NKX2-5 and* GATA3 *expression in the Grem1 knockout pylorus*
E18.5 pyloric sections of wild-type (A-D) or Grem1 knockout (E-H) mice shows that the expression of both NKX2-5 and Gata3 are relatively undisturbed in the absence of Grem1. In both genotypes, NKX2-5 is expressed throughout the ICM and OLM, while Gata3 expression is largely limited to the OLM.

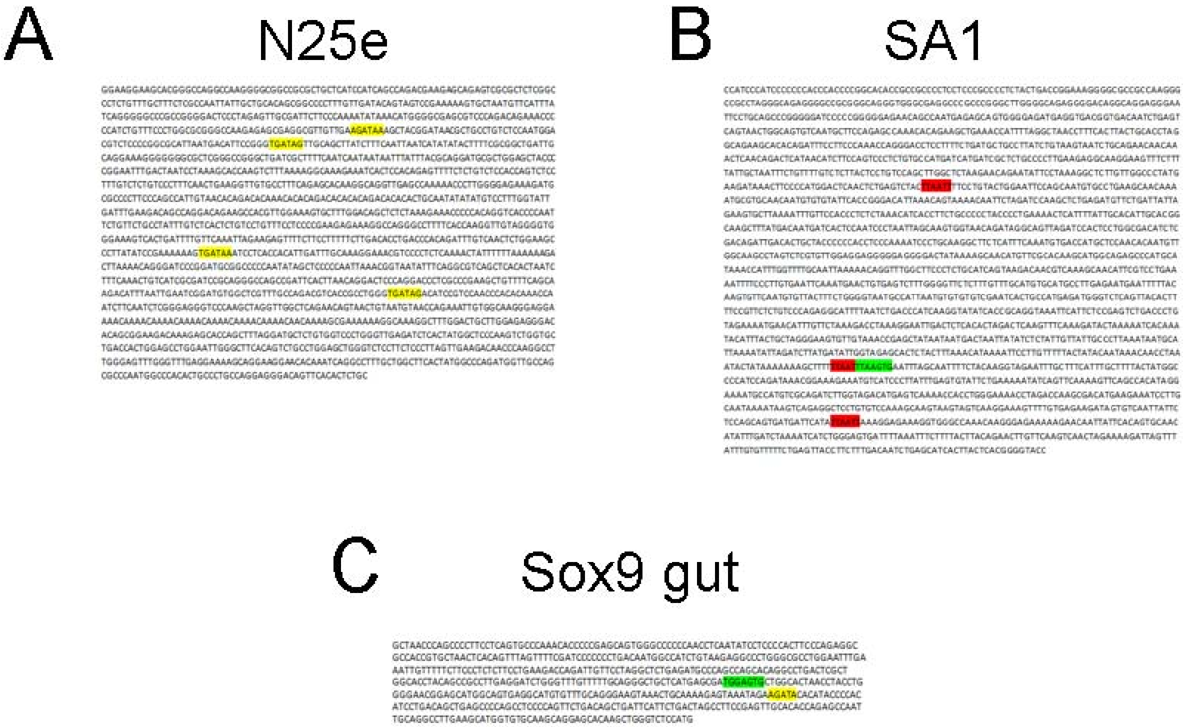

*Figure 4.3 Binding sites in pyloric enhancers*

Sequences for the pylorus specific enhancers of NKX2-5 (A), Gata3 (B), and SOX9 (C) illustrate few common motifs. The enhancers for both NKX2-5 and SOX9 display binding sites for Gata3 (yellow). The Gata3 and SOX9 enhancers both display binding sites for Nkx-25 (green), while only the Gata3 enhancer contains generic homeodomain binding sites.

### 4.3 Identification of pyloric targets of *Nkx2-5* and *Gata3*

While *Gata3* and *Nkx2-5* may not regulate one another, co-expression of *Gata3* or *Nkx2-5* in the pyloric OLM, and the remarkably similar phenotypes seen in the loss of either gene, suggests that they likely interact in some way in pyloric signaling. This may be through the independent regulation of common targets, or through the participation of both proteins in a transcriptional complex that might function to drive muscle differentiation. Since both genes encode transcription factors, one approach to explore this further would be to determine the direct downstream targets of each protein.

We could identify possible targets of these pyloric transcription factors using chromatin immunoprecipitation, followed by sequencing (ChIP-seq). This protocol allows us to uniquely identify occupied binding sites in potential pyloric target genes. Of particular interest would be finding targets that appear to have binding sites for both *Nkx2-5* and *Gata3*, since their phenotypes suggest that they could regulate common genes (indeed, we already know that they are both required for *Sox9* expression). To minimize noise within the assay, it would be ideal to assay only pyloric tissue. This could be accomplished through microdissection of the pylorus from the surrounding gastric and intestinal tissue. We could also use our existing *Nkx2-5*$^{\mathrm{eGFP/+}}$ or *Gata3*$^{\mathrm{eGFP/+}}$ reporter line and sort EGFP expressing cells for the ChIP analysis, limiting our analysis to only the *Nkx2-5* or *Gata3* positive cells at the pylorus. Narrowing the assay to this population would minimize noise, but will certainly limit the available tissue. These experiments are altogether feasible, since ChIP compatible antibodies for both genes have been previously tested [114, 139].

The binding sequences for NKX2-5 and GATA3 are well-described [4, 5]. NKX2-5 shows heterogenous binding affinity to a number of similar DNA sequences [4]. Among these, the highest affinity sequence is 5'-TNNAGTG-3'. Since NKX2-5 is a homeodomain protein [4], it also has a moderate affinity for the generic homeodomain sequence, 5'-TAAT-3'. Due to the commonplace nature of that short sequence, binding to similar sequences may or may not indicate pylorus specific NKX2-5 activation. The Gata family of transcription factors is known to bind to the sequence 5'-A/TGATAA/G-3', containing the central "GATA" motif for which the family is named. In silico analysis of the NKX2-5 and GATA3 targets implicated by this study will focus on these binding sites to determine potential gene targets for follow-up. To further narrow these potential targets, we will take advantage of microarray studies done earlier by our laboratory [7]. Those ChIP targets that are also clearly expressed at the pylorus will be prioritized.

### *Nkx2-5, Gata3, and Srf*

The independent regulation of *Gata3* and *Nkx2-5* is surprising in light of their common phenotype with gene deletion: both result in lack of smooth muscle differentiation of the pyloric OLM fascicle. One attractive hypothesis to bring these elements together is to consider the idea that GATA3, NKX2-5 and SRF work together in a complex that regulates smooth muscle genes. In this case, the loss of any of the three factors could result in failure of smooth muscle formation/differentiation. SRF is a well described regulator of cardiac and smooth muscle differentiation [140-142], including the differentiated gastrointestinal smooth muscle marker αSMA [143]. Interestingly, a tri-molecular complex of NKX2-5, GATA4 and SRF is thought to regulate the expression of cardiac α-actin [141]. NKX2-5 and GATA4 appear to act cooperatively and synergistically with SRF; interestingly, the DNA binding domain of NKX2-5 was dispensable in this function [141]. To determine if such a complex forms in pyloric muscle, we would assay NKX2-5, GATA3, and SRF for possible protein-protein interactions using co-immunoprecipitation assays (co-IP). Positive staining for GATA3 and NKX2-5 after the precipitation of SRF in pyloric tissue would strongly suggest that these genes cooperate in pyloric muscular regulation. Reporter assays for transcriptional activation in smooth muscle cells such as 10T1/2 cells could be used to further explore whether co-transfection of NKX2-5, GATA3 and/or SRF displays synergy in the regulation of smooth muscle genes such as αSMA and SM22α and to determine the domains of each factor that are required for these functions.

### 4.4 *Sox9* function at the pylorus

At least one target of both NKX2-5 and GATA3 may be *Sox9*. In this regard, it is interesting that a 516 bp sequence about 28 kb upstream of the *Sox9* locus appears to be responsible for SOX9 expression in the node, notochord, gut, bronchial epithelium, and pancreas [144, 145]. No other *Sox9* enhancer mapped to a gut region, suggesting that pyloric SOX9 expression is regulated by this region. Examination of this region appears to show a single binding site each for NKX2-5 and GATA3 (Figure 4.4). This implicates *Sox9* as an important convergence point for OLM development. Unfortunately, germline loss of SOX9 is incompatible with embryonic development [146]. Also, since SOX9 is expressed in both epithelium and mesenchyme at the pylorus, we cannot use a tool such as the CAGG-CreER mouse that we successfully employed for the conditional removal of *Nkx2-5*. Thus, investigating the role of

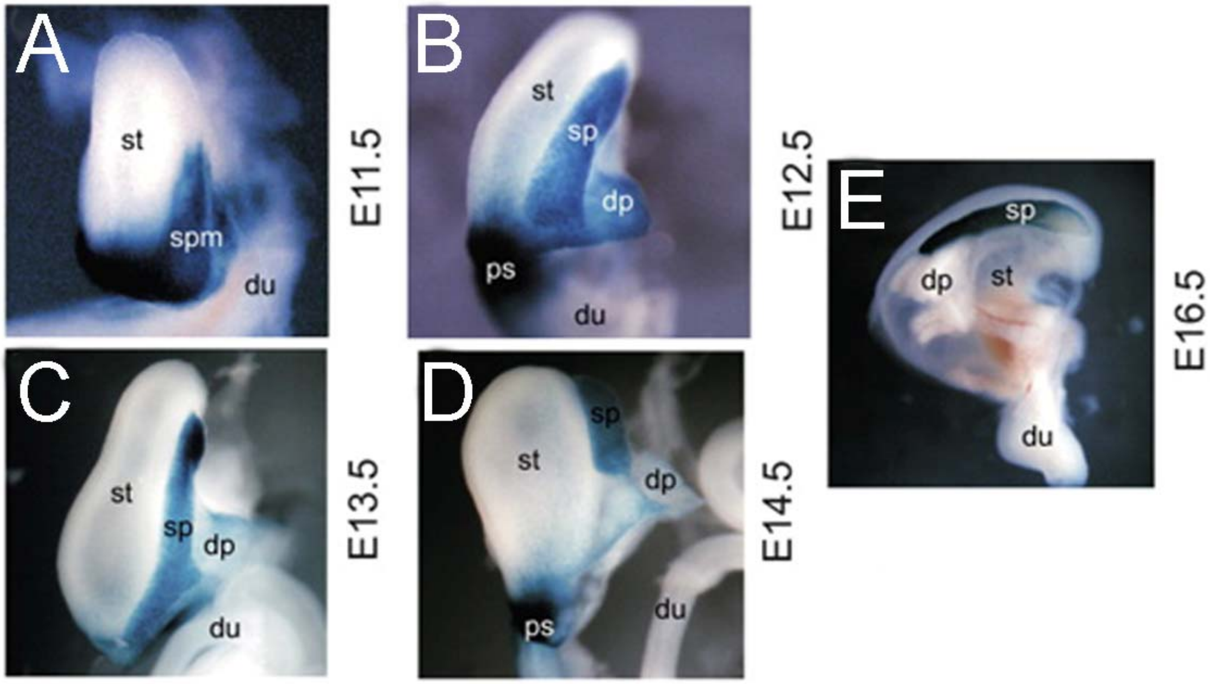

*Figure 4.4 Expression of the NGRS-lacZ gene*
X-gal staining of the NGRS-lacZ reporter mouse shows strong staining at the pylorus and the spleen. (A) At E11.5, the spleen is beginning to develop and shows a lacZ positive cell population moving to the lateral stomach from the pylorus. (B) By E12.5 there is an obvious splenic shape on the lateral stomach. The pylorus also shows strong NGRS expression at this timepoint. (C) The spleen narrows and the stream of cells from the pylorus grows more limited. (D) By E14.5 there are few cells still traveling from the pylorus to the spleen. (E) By E16.5, the splenic expression is completely distinct from that at the pylorus. Pyloric expression is hidden by pancreatic tissue (Figure adapted from Fig 1. in Burn et al. 2008 and printed with permission from Elsevier)

*Sox9* in pyloric function requires a mesenchyme-specific deletion of the gene. Since an *Nkx2-5* pylorus-specific enhancer has been defined [147](Figure 4.2), this region could be used to drive the expression of a pylorus-specific Cre. Using an existing *Sox9*$^{f/f}$ line, we could determine *Sox9*'s pyloric function.

Analysis of such a pylorus-specific *Sox9* knockout would proceed in a manner similar to that for the *Nkx2-5* and *Gata3* knockouts described in Chapter 2. It would be of interest to know whether there are changes in *Nkx2-5* and *Gata3* expression in the absence of *Sox9*. A microarray of the pylorus in the *Sox9* conditional knockout mouse would allow us to determine how genes are altered in this knockout, and thus identify some of the *Sox9* targets. We would expect this list to be a subset of the genes downstream of *Nkx2-5* and *Gata3* since pyloric *Sox9* expression is dependent on both those genes. It is interesting to recall that *Sox9* is expressed in a rather small subset of the cells that also express *Gata3* and *Nkx2-5*. Because so few cells in the center of the OLM domain express *Sox9*, it is tempting to speculate that *Sox9* could regulate a secreted signaling factor that could control the size of the OLM muscle. In this regard, it would be of interest to design a *Sox9* overexpressing mouse model to see if this might have an overly developed pyloric muscle, another possible model for IHPS.

### 4.5 Understanding the function of the pyloric cords

Thus far, I have described experiments that could extend our understanding of pyloric signaling, especially as it relates to *Nkx2-5*, *Gata3* and *Sox9*. However, the data in Chapter 3 present an interesting question which I have not yet addressed. Specifically, what is the function of the pyloric ligaments? While earlier works have addressed the presence of the pyloric ligaments [125], they did not present experimental evidence to support a hypothesis as to their function. Our data suggest that the pyloric cords are smooth muscle structures continuous with the pyloric OLM and express several of the same genes that are expressed in the OLM. Thus, we hypothesize that the OLM and pyloric ligaments form one continuous structure that may function to control the flexure and/or position of the antrum relative to the duodenum.

It would be of interest to determine whether in fetal or postnatal mice, disruption of these cords affects either the flexure of the stomach to the duodenum or the timing of the peristaltic contractions passing through this region. Thus, I would perform a series of experiments in E16.5 and P2 mice in which one or both of the ligaments are surgically cut. Such a study could be done by partial dissection (without removing the entire stomach and duodenum from the mouse), or in culture. We have previously described the successful culturing of short segments of intestine [148]. This protocol also works on early embryonic stomachs, allowing them to be cultured for a few days. Thus, we could culture E16.5 stomachs and observe the function of the ligaments. The ligaments could be induced to contract with the addition of neurotransmitters like acetylcholine, allowing the observation of the degree of flexure. Flux of a fluorescent dye injected into the stomach coud also be studied. A matched control mouse in which the abdominal cavity is opened but the cords are not severed would be used as a control to determine what normal flexure and flux should be in those situations. While such a culture system allows a greater deal of freedom in manipulation of the target organ, it is also a much more artificial system since there is no real food movement through the GI tract before birth. One additional caveat to these experiments is the substantial innervation of the pyloric region, most of which is inhibitory. Thus, dissection of this region has the potential to alter peristalsis by itself, making these experiments somewhat technically challenging

## 4.6 Conclusion

The data presented in this thesis provide the first cellular and molecular characterization of the development of the pyloric OLM and the pyloric ligaments. These data also extend our current understanding of pyloric signaling by identifying the functions of *Nkx2-5* and *Gata3* at the mammalian pylorus. Since, at the pylorus, *Nkx2-5* is expressed earlier and more widely than *Gata3*, we initially speculated that *Nkx2-5* was an important regulator of *Gata3*. When we found that the two knockouts displayed highly similar phenotypes (see Chapter 2), we were even more ready to see that *Nkx2-5* was upstream of *Gata3*. Yet, confounding expectations, the data indicated that *Nkx2-5* does not regulate *Gata3* expression at the pylorus. Indeed, our data suggest that the upstream regulation of these two genes during the course of pyloric development is independent. The idea that both genes could function in a similar pathway, perhaps in conjunction with SRF, could provide a unifying hypothesis to explain the similar phenotypes, linking the disparate muscular phenotypes into one coherent signaling tree. The expansion of this tree and the incorporation of previous data sets, like the *Barx1*, *Bapx1*, and *Six2* studies, would greatly extend our current understanding of pyloric development and help to uncover the molecular defects responsible for idiopathic hypertrophic pyloric stenosis.

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