

## **Loss of GATA4 causes ectopic pancreas in the stomach**

Running title: **Pancreatic heterotopia in *Gata4*-deficient mice**

Authors: Elisa Rodríguez-Seguel<sup>1§</sup>, Laura Villamayor<sup>1§</sup>, Noelia Arroyo<sup>1</sup>, Mónica Pérez<sup>3</sup>, Francisco X. Real<sup>3,4,5</sup>, Franz Martín<sup>1,2</sup>, David A. Cano<sup>6</sup> and Anabel Rojas<sup>1,2\*</sup>

### **Affiliations:**

<sup>1</sup>Centro Andaluz de Biología Molecular y Medicina Regenerativa-CABIMER, Universidad Pablo de Olavide, Universidad de Sevilla, Consejo Superior de Investigaciones Científicas (CSIC), Seville, Spain

<sup>2</sup>Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Madrid, Spain

<sup>3</sup>Centro Nacional de Investigaciones Oncológicas (CNIO), Madrid, Spain

<sup>4</sup> CIBERONC, Spain

<sup>5</sup> Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain

<sup>6</sup> Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Seville, Spain

\*Corresponding author

Avda. Américo Vespucio s/n.

Parque Científico Isla de la Cartuja

41092 Sevilla, Spain

E-mail: [anabel.rojas@cabimer.es](mailto:anabel.rojas@cabimer.es)

Phone: (+34) 954 467 427

FAX: (+34) 954 461 664

§: These authors contributed equally to this study.

The authors have declared that no conflict of interest exists.

## **Abstract**

Pancreatic heterotopia is defined as pancreatic tissue outside its normal location in the body and anatomically separated from the pancreas. In this work we have analyzed the stomach glandular epithelium of *Gata4<sup>fllox/fllox</sup>; Pdx1-Cre* mice (*Gata4KO* mice). We found that *Gata4KO* glandular epithelium display an atypical morphology similar to the cornified squamous epithelium and exhibit upregulation of forestomach markers. The developing gastric units fail to form properly and the glandular epithelial cells do not express markers of gastric gland in the absence of GATA4. Interestingly, the developing glands of *Gata4KO* stomach express pancreatic cells markers.

Furthermore, a mass of pancreatic tissue located in the subserosa of *Gata4KO* stomach is observed at adult stages. Heterotopic pancreas found in *Gata4*-deficient mice contains all three pancreatic cell lineages, ductal, acinar and endocrine cells. Moreover, *Gata4* expression is downregulated in ectopic pancreatic tissue of some human biopsy samples.

Key words: GATA4 transcription factor, pancreatic heterotopia, stomach development, glandular stomach, pancreas.

## **Introduction**

Cell fate allocation during embryonic development is controlled by complex developmental programs that involve a network of signaling pathways and transcription factors. Deregulation of these developmental programs, including defects in organ patterning, morphogenesis or cell differentiation, might cause the formation of organs at ectopic locations.

Heterotopic pancreas is defined as pancreatic tissue outside its usual anatomical location but without anatomic or vascular connection to the normal pancreas. Pancreatic heterotopia is rare. In humans, ectopic pancreatic tissue is mainly found in the distal stomach and duodenum and at lower frequency in the esophagus, ileum and biliary tree [1]. Though mostly asymptomatic, this condition might result in pancreatitis, ulcerations, and gastric obstruction and, in rare cases, malignant degeneration [2]. The precise pathogenic mechanism of ectopic pancreas in human is poorly understood. Given the proximity of the pancreas to the stomach and duodenum along the primitive foregut, it has been proposed the misplacement of pancreatic cells into the developing gastrointestinal system as a hypothesis for pancreatic heterotopia [1]. An alternative hypothesis implies a metaplastic process in which ectopic pancreatic tissue would arise from other endodermal areas different from the pancreatic endoderm that undergo pancreatic metaplasia migrating to the submucosa of the gut during embryogenesis [3]. A third theory is based on the existence of totipotent cells lining the endoderm that differentiate into pancreatic tissue [4].

Several studies in mice have directly associated the formation of ectopic pancreas with defects in embryonic signaling pathways important for pancreas organogenesis. Thus, loss of *Hes1*, a Notch signaling transducer, leads to heterotopic pancreas in the stomach, duodenum and common biliary duct [5,6]. This seems to be due to defects in morphogenesis of the dorsal pancreas primordium which ingresses into the stomach domain during embryonic development [7]. Inhibition of Hedgehog signaling in the foregut also induces pancreatic heterotopia in stomach and duodenum [8,9]. Finally, genetic misexpression of the basic helix-loop-helix transcription factors *Ptf1a* in the *Pdx1* expression domain of the foregut endoderm causes conversion of glandular stomach, anterior duodenum and the extra-hepatic biliary system into pancreas highlighting the critical role of this transcription factor in defining pancreas allocation in the foregut endoderm [10-12].

In the mouse, the stomach is divided into the forestomach (which it is not found in humans) that comprises a stratified squamous epithelium and the hindstomach or glandular stomach. The glandular stomach is composed of columnar epithelial cells and can be further divided into the corpus and the most posterior region, the antrum [13-15]. The stomach derives from the foregut endoderm, which also gives rise to pancreas among other endodermal-derived organs. Although far from being completely understood, the specification of the embryonic stomach is regulated by a number of signaling pathways (such as WNT, SHH and BMP) and transcription factors [14]. The regionalization of the gut endoderm is achieved in part by restricting the expression of transcription factors to particular domains. Thus, the foregut endoderm expresses SOX2 while the posterior region or hindgut expresses CDX2 [13-15]. Once the gastric identity is specified, the stomach undergoes further regionalization, a process that also involves

selective expression of specific transcription factors in these areas. For example, *Pdx1* is expressed in the foregut region that gives rise to antrum and pylorus (in addition to pancreas, anterior duodenum and proximal extrahepatic biliary system) [16]. By contrast, *Sox2* expression is maintained at much higher levels in the forestomach [17].

GATA4 is a member of the GATA zinc-finger transcription factor family that control specification, differentiation and proliferation of various mesoderm- and endoderm- derived cell types [18-22]. In the fetal stomach, *Gata4* is expressed in the epithelium of the hindstomach and duodenum, and in adult stomach it is localized in pit, neck and parietal glandular epithelial cells [23-26] suggesting a role in glandular stomach formation. Indeed, studies using chimeric embryos, to circumvent the early lethality of *Gata4* null mice [27-29] lends support to this notion. *Gata4*-null stomach cells fail to express markers associated with differentiation of gastric epithelial cell lineages [25]. However, it remains unclear whether these defects in glandular stomach cell differentiation reflect a cell-autonomous role of GATA4.

GATA4 has been shown to control cell identity during embryonic development by activating or repressing key regulators of endodermal fates. Thus, mice lacking GATA4 in the jejunum lost jejunal identity and fates into ileum lineage [30]. Interestingly, simultaneous inactivation of GATA4 and GATA6 results in dorsal and ventral pancreatic progenitors acquiring stomach and intestinal and lineages, respectively [31]. GATA4 is a key player in pancreas organogenesis. Mutations in *GATA4* result in severe hypoplasia or complete pancreatic agenesis in humans [32]. In stark contrast, conditional inactivation of *Gata4* in pancreatic progenitors does not have any apparent effect on pancreas formation in mice. However, mice with simultaneous

inactivation of *Gata4* and *Gata6* fail to develop pancreas and died shortly after birth with hyperglycemia [33,34] indicating functional redundancy of these transcription factor in mouse development.

In this work we have analyzed the stomach glandular epithelium of *Gata4<sup>fllox/fllox</sup>*; *Pdx1-Cre* mice (*Gata4KO* mice, hereafter). We found that *Gata4KO* glandular epithelium display an atypical morphology similar to the cornified squamous epithelium. The developing gastric units fail to form properly and the glandular epithelial cells do not express markers of gastric gland in the absence of GATA4. Interestingly, the developing glands of *Gata4KO* stomach express pancreatic cells markers and a mass of pancreatic tissue located in the subserosa of *Gata4KO* stomach is observed at adult stages. Heterotopic pancreas found in *Gata4*-deficient mice contains all three pancreatic cell lineages, ductal, acinar and endocrine cells. Analysis of ectopic pancreas in human biopsies revealed a downregulation of *Gata4* expression in some of the samples analyzed.

## MATERIAL AND METHODS

### Mice

*Gata4<sup>flox/flox</sup>*, *Pdx1-Cre*, *ROSA26ReYFP* and *Ptf1a-Cre* mice and the strategies for genotyping have been previously described [33,35-38]. All experiments using animals complied with institutional guidelines, and were reviewed and approved by the Institutional Animal care and Use Committee (IACUC) of the University of Sevilla, Spain or the Instituto de Salud Carlos III and Comunidad Autónoma de Madrid.

### Histology, Immunohistochemistry and Immunofluorescence.

Dissected pancreata collected at adult stages were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, dehydrated with ethanol and xylene, and embedded in paraffin. Histological analyses were performed as previously described [39,40]. The following primary antibodies were used at the indicated dilution: mouse anti-GATA4 (1:100 Santa Cruz, Sc-25310); rabbit anti-Gastrin (1:50 Abcam, ab8492); mouse anti-alpha smooth muscle actin (1:300, Sigma-Aldrich, A5228); mouse anti-Hydrogen Potassium ATPase Beta (1:2000 Abcam, ab2866), rabbit anti-Sox2 (1:500 Abcam 97959), goat anti-GFP (1:200 Abcam, ab6673); guinea pig anti-Pdx1 (1:200 Abcam, ab47308); mouse anti-insulin (1:500 Sigma-Aldrich, I2018); mouse anti-E-Cadherin (1:100, BD Bioscience, 61018); rabbit anti-Ptf1a (1:2000, Beta Cell Biology Consortium, AB2153); mouse anti-amylase (1:200 Santa Cruz, Sc-46657); mouse anti-MUC5AC (1:200 Thermo Scientific, MS-145-P); mouse anti-Glucagon (1:200, Sigma-Aldrich, G2654); rabbit anti-MafA (1:50, Bethyl laboratories, A300-611A); rabbit anti-Glut2 (1:400, Millipore, 07-1402); rabbit anti- HNF1 $\beta$  (1:100, Santa Cruz Sc-8986), Biotinylated *Dolichos biflorus* Agglutinin (DBA) (1:300; Vector Laboratories, B-1035), rabbit anti-cytokeratin 14 (1:200, Abcam, ab15461); rabbit anti-Trp63 (1:500, Abcam, ab53039). All pictures from immunofluorescence and

immunohistochemistry shown are representative of at least 6 independent samples of the indicated genotype.

### **Human Samples**

Gastric biopsies were obtained upon obtaining an informed consent from each patient. The study protocol was approved by the ethics committee of the Hospital Universitario Virgen del Rocío (HUVR), Seville and the study was conducted according to the principles of the Declaration of Helsinki. Immunofluorescence for GATA4 was performed on 4  $\mu\text{m}$  paraffin sections of human stomach from twenty-four patients. All samples analyzed contained pancreatic tissue located in the subserosa layer of the stomach. Eighteen samples out of the twenty-four also contained glandular stomach or proximal duodenum.



## Results

### *Morphological and cell differentiation defects in glandular stomach in the absence of Gata4*

To investigate the role of GATA4 on the formation of the glandular stomach, mice with a conditional (flox) allele of *Gata4* (*Gata4<sup>flox/flox</sup>*) were crossed with mice expressing the Cre recombinase under the control of the pancreatic and duodenal homeobox gene 1 (*Pdx1*) promoter (*Pdx1-Cre* mice) [36]. PDX1 is expressed in all pancreatic progenitors but is also expressed in the caudal region of the developing stomach as well as the duodenum, where it overlaps with GATA4 (Fig. 1A-C). To monitor Cre recombinase activity, *Pdx1-Cre* and *Gata4<sup>flox/flox</sup>; Pdx1-Cre* mice were crossed to a *ROSA26ReYFP* reporter strain [37]. Immunofluorescence analyses of E17.5 embryos revealed robust *eYFP* expression in the antrum of both *Pdx1-Cre*; *ROSA26ReYFP* (Figure 1D, F) and *Gata4<sup>flox/flox</sup>; Pdx1-Cre*; *ROSA26eYFP* embryos (Figure 1E, G). Scattered eYFP-positive cells were also observed in the corpus region along the greater curvature of the stomach in both mouse strains (Figure 1D, E, H, I). In agreement with this *Cre* expression pattern, efficient GATA4 loss was observed in the antrum of *Gata4<sup>flox/flox</sup>; Pdx1-Cre*; *ROSA26ReYFP* embryos while a mosaic pattern of GATA4 protein is observed in the corpus (Fig. 1G, I). A similar result was obtained by immunohistochemistry analyses in *Gata4<sup>flox/flox</sup>; Pdx1-Cre* (hereafter, *Gata4KO* mice) and control embryos (*Gata4<sup>flox/+</sup>; Pdx1-Cre* or *Gata4<sup>flox/flox</sup>*) (Fig. 1J-M).

We next analyzed whether the lack of GATA4 affects stomach development by analyzing the stomach epithelium at various embryonic stages. No major morphological abnormalities were observed at embryonic day (E) 13.5 and E15.5 (Supplemental Fig. 1). However, by E17.5, *Gata4KO* embryos failed to form the characteristic developing

gastric units of normal stomach (Supplemental Figure 1). In newborn mice, *Gata4KO* stomach lacked gastric glands and the epithelium appeared mostly flattened (Fig. 2B, D) in contrast to control stomach in which invaginations of the gastric epithelium were clearly observed (Fig. 2A, C). The stomach of adult (2-3-month-old) control mice displayed a well-defined and highly organized glandular epithelium (Fig. 2E, G). In stark contrast, *Gata4*-deficient antrum showed an atypical morphology with appearance of a cornified squamous epithelium (Fig. 2F, H). To determine whether glandular cell differentiation was impaired in *Gata4KO* stomach, we analyzed several markers of differentiated gastric cells. At E17.5, parietal cells expressing the gastric hydrogen potassium *ATPase* ( $\text{HK}^+$ -ATPase) in the corpus and gastrin-expressing enteroendocrine cells in the antrum were easily observed in the developing glands of control mice (Supplemental Fig.1). However, only few  $\text{HK}^+$ -ATPase-expressing cells were found in the corpus of *Gata4 KO* embryos, likely due to mosaic Cre activity observed in this region of the stomach (Supplemental Fig.1). No gastrin-expressing cells were observed in the antrum epithelium of the *Gata4KO* embryos (Supplemental Figure 1). At both P1 and adult stomach, well-differentiated parietal cells (positive for  $\text{HK}^+$ -ATPase) (Fig. 2I, K), mucus-producing cells (positive for MUC5AC) (Fig. 2M, O) and G-enteroendocrine cells (positive for Gastrin) were found in control mice (Fig. 2Q, S). In stark contrast, the expression of  $\text{HK}^+$ -ATPase, MUC5AC and Gastrin were dramatically reduced in the glandular epithelium of *Gata4* knockout mice (Fig. 2J, L, N, P, R, T). These results indicate that GATA4 plays a critical role in the differentiation of the gastric epithelium.

***Upregulation of squamous epithelial markers in the corpus and antrum of *Gata4KO* mice***

The cornified appearance of the corpus and antrum of *Gata4*-deficient mice, prompted us to investigate the expression of squamous epithelial markers. Cytokeratin 14 and the transcription factor TRP63 are expressed in forestomach but not in corpus and antrum. As expected, immunofluorescence analyses for these squamous epithelial markers revealed a clear boundary between the forestomach and corpus in newborn (Fig. 3A, A', C, C') and adult control mice (Fig. 3G, G', I, I'). However, this boundary was not apparent in *Gata4KO* stomach and both the antrum and corpus showed a marked increase in cytokeratin 14 (Fig. 3B, B', B'', H, H') and TRP63 (Fig. 3D, D', D'', J, J') immunostaining. The gastric SRY-related HMG transcription factor SOX2 was strongly expressed in the forestomach whereas is much lower expressed in both corpus and antrum of newborn (Fig. 3E, E'), and adult control stomach (Fig. 3K, K'), as previously reported [13]. However, a dramatic upregulation of SOX2 expression was observed in the corpus and antrum of newborn (Fig. 3F, F', F'') and adult *Gata4KO* mice (Fig. 3L, L'). Altogether, these results demonstrate that GATA4 is required to maintain glandular stomach identity.

### ***Ectopic pancreas in Gata4-deficient mice***

Histological analyses of embryonic stomach at E17.5 revealed an ingression of the flattened epithelium of embryonic *Gata4KO* antrum into the subserosa layer of the stomach. Remarkably, the invaginated gastric epithelium formed a dilated duct structure adjacent to clusters of cells with pancreatic acinar morphology (Fig. 4D). By contrast, control stomach displayed normal developing unit glands (Fig. 4A). At P1 and in adult stomach of *Gata4KO* mice, this ectopic pancreatic tissue expanded and it was well-distinguishable by hematoxylin and eosin staining (Fig. 4G, J, M). The penetrance of the ectopic pancreas phenotype in all analyzed neonates and adult *Gata4KO* mice was

100% (20 neonates and 17 adult mice in total), while it was never observed in control mice (>50 analyzed mice). *Gata4<sup>fllox/fllox</sup>; Pdx1-Cre ROSA26R-eYFP* mice were used to better visualize the ectopic pancreatic tissue. In control *Pdx1-Cre ROSA26R-eYFP* embryonic stomach, eYFP-labelled cells were only observed in the developing glands (Fig. 4B). However, eYFP-labelled *Gata4* knockout cells in the subserosa layer of the stomach appeared continuous with the gastric epithelium, discontinuing the submucosal and muscular layer, as revealed by smooth muscle  $\alpha$ -actin immunostaining (Fig. 4E, H, K). Interestingly, cells from PDX1-positive ducts located in the subserosa layer of the stomach (Fig. 4F, L) clustered into acinar-like structures that expressed amylase (Fig. 4F, I, N) and the acinar transcription factor PTF1A (Fig. 4O). No immunostaining for amylase was observed in the control stomach (Fig. 4C). Remarkably, endocrine cells expressing insulin and glucagon were also detected in the ectopic pancreas in *Gata4KO* stomach (Fig. 4P). The distribution of endocrine cells in the ectopic pancreas resembled murine pancreatic islets, with a core of insulin-positive cells surrounded by glucagon-positive cells (Fig. 4P). Moreover, insulin-producing cells expressed the mature  $\beta$  cell markers MAFA (Fig. 4Q) and the glucose sensor GLUT2 (Fig. 4R).

#### ***Gata4-deficient gastric epithelium expresses pancreatic markers***

Ectopic pancreas has been associated to impaired Notch signaling in mice [5-7]. We examined the expression of *Hes1*, a mediator of Notch signaling, and its downstream target *Ngn3* in E13.5 and E15.5 *Gata4KO* pancreas and stomach. Quantitative RT-PCR analysis showed similar *Hes1* and *Ngn3* expression levels in *Gata4KO* pancreas compared to control pancreas at both stages (Supplemental Figure 2). Similarly, *Hes1* and *Ngn3* expression was not altered in the absence of GATA4 activity in the stomach (Supplemental Figure 2). Moreover, in contrast to what it is

observed in *Hes1* mutant embryonic pancreata, the dorsal pancreas of *Gata4* KO mice displayed normal morphology and the pancreatic epithelium, labeled by E-cadherin, did not contact the stomach epithelium, (Supplemental Figure 2). These results indicate that the formation of ectopic pancreas in *Gata4KO* embryos is not due to decreased Notch signaling. Inhibition of Hedgehog signaling in the early foregut endoderm has also been associated with pancreatic heterotopia in stomach [8,9]. However, the gastric epithelium in the *Gata4KO* antrum showed a marked upregulation of *Shh* expression (Supplemental Figure 2), and therefore it does not support a role for loss of Hedgehog signaling in pancreatic heterotopia of *Gata4* KO mice. Indeed, our results are in agreement with a previous study showing that GATA4 attenuates *Shh* expression in the distal stomach [25].

To determine whether loss of GATA4 specifically in pancreatic progenitors leads to the formation of ectopic pancreas in a wildtype stomach, we crossed *Gata4* floxed mice with *Ptf1a-Cre* mice [38]. *Ptf1a-Cre* is active in pancreatic cells from E9.5 onwards although it is later restricted to the acinar cell lineage. However, it is not expressed in the stomach. Accordingly, strong GATA4 expression was observed in the glandular stomach of *Gata4<sup>flox/flox</sup>; Ptf1a-Cre* mice (Supplemental Figure 3). Histological and immunohistochemical analyses revealed well-differentiated gastric unit glands in *Gata4<sup>flox/flox</sup>; Ptf1a-Cre* mice (Supplemental Figure 3). Furthermore, no ectopic pancreatic tissue was observed in any of the *Gata4<sup>flox/flox</sup>; Ptf1a-Cre* conditional knockout mice analyzed (n=10). These data indicate that the absence of GATA4 in the pancreatic compartment does not induce the formation of ectopic pancreatic tissue in the stomach. The morphological analysis of the developing gastric antrum of *Gata4<sup>flox/flox</sup>; Pdx1-Cre* embryos suggests that the ectopic pancreas originates directly

from the glandular epithelium rather than from the migration of pancreatic cells into the stomach antrum during development (Figure 4E, H). To test this hypothesis, we analyzed the gastric epithelium of *Gata4*-deficient embryos for expression of pancreatic markers. As expected, the developing gastric glands and proximal duodenum in control embryos strongly expressed *Pdx1* (Fig. 5A, C) but no insulin-positive endocrine cells were found. In stark contrast, scattered insulin-positive cells were observed in the glandular stomach and in the duodenum of the *Gata4*-deficient glandular epithelium (Fig. 5B, D). Moreover, an upregulation of the ductal marker HNF1 $\beta$  is observed in areas of *Gata4* knockout glandular epithelium that invaginates into the subserosa layer, discontinuing the smooth muscle layer (Fig. 5F). Interestingly, the HNF1 $\beta$ -positive cells strongly react with *Dolichos biflorus agglutinin (DBA)* (Fig. 5H) which selectively labels ductal cells in the pancreas [41]. No upregulation of HNF1  $\beta$  was observed in the glandular epithelium of control embryos (E, G).

### ***GATA4 is downregulated in human heterotopic pancreas***

To investigate the potential role of GATA4 in human pancreatic heterotopia, we compared its expression in samples of 24 patients containing ectopic pancreatic tissue and in normal pancreatic tissue. In 8 of the cases, strong GATA4 immunostaining was detected in the acinar cells of the ectopic pancreas, similar to the immunostaining observed in normal pancreas (Fig. 6B, G). However, 16 samples displayed low accumulation of GATA4 protein in ectopic pancreas compared to normal, orthotopic pancreas (Fig. 6L). Of note, all analyzed samples showed strong immunostaining for the acinar marker amylase serving as an internal control for adequate immunohistochemical analysis (Fig. 6E, J, O). Out of the 24 samples from patients containing heterotopic pancreas, 18 also contained glandular stomach/proximal duodenum, which allowed us

to evaluate GATA4 expression in both tissues of the same patient. Remarkably, the samples with weak GATA4 immunostaining in the ectopic pancreas also displayed weak GATA4 immunostaining in the glandular stomach and proximal duodenum (Fig. 6D, I, N). This result might suggest that, at least in some cases, GATA4 downregulation might be associated with formation of ectopic pancreas in humans.

## Discussion

Here we show that the transcription factor GATA4 is critical for the maintenance of cell identity in the hindstomach and the differentiation of glandular epithelial cells. The inactivation of *Gata4* in the stomach using the *Pdx1-Cre* driver mouse strain, impairs glandular epithelium formation and leads to aberrant induction of forestomach markers in the glandular stomach. More remarkably, *Gata4* deficiency causes ectopic pancreas in the subserosa layer of the stomach.

Our results provide support to previous studies that have suggested an important role for GATA4 in glandular stomach development [24,25]. GATA4 is expressed in the hindstomach but not in the forestomach during embryonic development. Studies on chimeric embryos have shown that *Gata4*-null cells contribute to the squamous gastric epithelium but not to the glandular epithelium. However, these studies could not distinguish whether GATA4 was necessary for gastric specification and/or differentiation. We used the *Pdx1-Cre* mouse line to inactivate *Gata4*, which targets the antral region precursors and, less efficiently in a mosaic fashion, the corpus. Therefore, inactivation of *Gata4* in using *Pdx1-Cre* driver mice would occur only after these regions have been specified. Further studies inactivating *Gata4* in the foregut epithelium would be necessary to establish the role of GATA4 in stomach specification. Although we could not properly examine the role of GATA4 in this process, our results demonstrate that GATA4 is necessary for glandular stomach cell differentiation. Thus, the expression of markers of parietal cells, enteroendocrine, and mucus-producing cells is dramatically reduced in the *Gata4KO* stomach. In agreement with our findings, it was shown in the above study that the scarce *Gata4*-null cells incorporated into the chimeric glandular stomach lacked expression of gastric markers. Interestingly, we found that



*Gata4KO* embryos exhibit a transformation of the hindstomach from columnar to a stratified squamous type epithelial morphology. Notably, increased expression of forestomach epithelium markers including the transcription factor SOX2, cytokeratin 14 and TRP63 were observed in the antral and corpus region of *Gata4KO* stomach suggesting that loss of GATA4 might lead to anteriorization of the hindstomach. These data suggest that GATA4 maintains glandular stomach cell identity by repressing markers of the anterior stomach. This role is reminiscent of the role of GATA4 in regulating the jejunal-ileal boundary in the small intestine [42,43]. The exact mechanism by which GATA4 maintains hindstomach identity remains to be determined. However, loss of GATA4 results in aberrant *Sox2* expression in the glandular stomach and SOX2 plays a key role in gastric epithelial differentiation. Indeed, misexpression of *Sox2* in the posterior region of the primitive gut induces a gastric-like phenotype in the intestine [44]. Thus, it is tempting to speculate whether GATA4 might directly repress *Sox2* expression in glandular stomach.

Unexpectedly, *Gata4KO* mice exhibited pancreatic heterotopia in the stomach. Cells of all pancreatic lineages (acinar, endocrine and ductal) were found in *Gata4KO* stomach. Importantly, ectopic pancreas was only observed in the hindstomach or in the proximal duodenum. The molecular determinants of pancreatic heterotopia and the pathogenic mechanisms involved are not well known. Earlier studies suggest that pancreatic heterotopia in mice with mutations in *Hes1* was caused by transdifferentiation of the embryonic stomach to pancreatic tissue [6], but a recent study proposes that aberrant morphogenesis of the dorsal pancreas primordium is the underlying mechanism in *Hes1* mutant mice [7]. We did not observe changes in *Hes1* and *Ngn3* (a HES1 target gene) expression in the dorsal pancreas of *Gata4KO* embryos,

suggesting that the heterotopic pancreas observed in *Gata4*-deficient mice might be independent of Notch signaling.

Although we cannot conclusively identify the basis for the pancreatic heterotopia in *Gata4KO* mice, our observations provide evidence, albeit circumstantial, for a transcommitment of regions of the prospective glandular stomach to pancreatic lineage. The inactivation of *Gata4* exclusively in the pancreatic progenitors using a highly pancreas-specific Cre mouse line (*Ptf1a-Cre*) did not lead to the formation of ectopic pancreas, suggesting that the absence of GATA4 in the stomach domain is selectively required for this embryonic disorder. The presence of scattered insulin positive cells and the upregulation of HNF1 $\beta$  ductal marker in the developing unit glands of *Gata4*-deficient mice might suggest that endodermal tissue migrates to the submucosa during embryogenesis transforming into pancreatic tissue. However, this theory fails to explain why full tissue conversion is not observed in the entire *Pdx1* positive domain of the glandular stomach. Further studies will be required to determine the exact mechanism. Nevertheless, our findings point to an important role of GATA4 in establishing cell lineages during foregut embryonic development. Indeed, a recent study supports a role for GATA factors in the foregut patterning. Simultaneous inactivation of *Gata4* and *Gata6* results in pancreatic agenesis [33,34]. Further studies in the embryonic pancreatic rudiments of these double mutants have shown a cell fate switch in dorsal and ventral pancreas to stomach and intestine, respectively [31]. However, individual *Gata4KO* embryos were not analyzed in this study.

In humans, pancreatic heterotopia is most commonly observed in stomach and intestine and, more rarely, in esophagus, biliary tract and spleen. We examined GATA4

expression in a series of human gastric biopsies with pancreatic heterotopia. Although far from conclusive, a substantial number of these samples showed decreased GATA4 expression in the ectopic pancreas suggesting that GATA4 might be associated with pancreatic heterotopia in humans as well. Interestingly, decreased GATA4 expression in ectopic pancreas correlates with decreased GATA4 expression in the glandular stomach and proximal duodenum. These results, together with our observations inactivating *Gata4* using the *Ptfla-Cre* line, support the idea that specific loss of GATA4 in the stomach/duodenum causes the formation of ectopic pancreas.

Our findings raise the question as to whether mutations in *GATA4* (or other genes regulating *GATA4* expression) might be linked to pancreatic heterotopia in humans. Patients with *GATA4* mutations have been reported. Indeed, a recent study has reported that mutations in *GATA4* are a cause of neonatal or childhood-onset diabetes [32]. However, no analysis of the stomach in these patients has been described and deeper imaging studies of these patients might be warranted.

#### **Acknowledgements:**

We thank Antonio Cárdenas and Ana Belén Hitos for their technical assistance. E. R-S. was supported by a “Juan de la Cierva” posdoctoral fellowship from Spanish Ministry of Economy and Competitiveness (IJCI-2014-19251). L.V. was supported by a contract from Spanish Ministry of Economy and Competitiveness (RYC-2013-14533). This work was supported by grants from the Spanish Ministry of Economy and Competitiveness BFU2017-82497-P to AR, ISCIII-Subdirección General de Evaluación y Fomento de la Investigación co-funded with Fondos FEDER, PI16/00175 to D.A.C.,

the Andalusian Ministry of Science and Innovation CTS-7478 to D.A.C) and grant SAF2015-70553-R to FXR. CNIO is supported by the “Ministerio de Ciencia, Innovación y Universidades” as a Centro de Excelencia Severo Ochoa (SEV-2015-0510). This research has been conducted using samples from the HUVR-IBiS Biobank (Andalusian Public Health System Biobank and ISCIII-Red de Biobancos PT17/0015/0041). The authors would like to thank Carolina Castilla and Fernando Carmona (HUVR-IBiS Biobank) for their outstanding technical help.

### **Author contributions**

E.R.S., L.V., N, A., M.P. conceived and carried out experiments. F.M, F.X.R., D.A.C, and A.R participated in the design of the study, conceived and analyzed the data. D.A.C. and A.R. wrote the manuscript. All authors reviewed the paper and had final approval of the submitted manuscript.

## References

1. Kung JW, Brown A, Kruskal JB, *et al.* Heterotopic pancreas: typical and atypical imaging findings. *Clin Radiol* 2010; **65**: 403-407.
2. Cazacu IM, Luzuriaga Chavez AA, Noguerras Gonzalez GM, *et al.* Malignant Transformation of Ectopic Pancreas. *Dig Dis Sci* 2019; **64**: 655-668.
3. Park E, Kim H, Jung KW, *et al.* Heterotopic pancreas in omphalomesenteric duct remnant results in persistent umbilical discharge. *Korean J Pathol* 2014; **48**: 323-326.
4. Stock C, Keutgen XM, Pisapia D, *et al.* Heterotopic pancreatic neoplasm presenting as an obstructing mass at the fourth portion of the duodenum. *JOP* 2011; **12**: 241-243.
5. Sumazaki R, Shiojiri N, Isoyama S, *et al.* Conversion of biliary system to pancreatic tissue in Hes1-deficient mice. *Nat Genet* 2004; **36**: 83-87.
6. Fukuda A, Kawaguchi Y, Furuyama K, *et al.* Ectopic pancreas formation in Hes1 -knockout mice reveals plasticity of endodermal progenitors of the gut, bile duct, and pancreas. *J Clin Invest* 2006; **116**: 1484-1493.
7. Jorgensen MC, de Lichtenberg KH, Collin CA, *et al.* Neurog3-dependent pancreas dysgenesis causes ectopic pancreas in Hes1 mutant mice. *Development* 2018; **145**.
8. Hebrok M, Kim SK, Melton DA. Notochord repression of endodermal Sonic hedgehog permits pancreas development. *Genes Dev* 1998; **12**: 1705-1713.
9. Kawahira H, Ma NH, Tzanakakis ES, *et al.* Combined activities of hedgehog signaling inhibitors regulate pancreas development. *Development* 2003; **130**: 4871-4879.
10. Afelik S, Chen Y, Pieler T. Combined ectopic expression of Pdx1 and Ptf1a/p48 results in the stable conversion of posterior endoderm into endocrine and exocrine pancreatic tissue. *Genes Dev* 2006; **20**: 1441-1446.
11. Jarikji ZH, Vanamala S, Beck CW, *et al.* Differential ability of Ptf1a and Ptf1a-VP16 to convert stomach, duodenum and liver to pancreas. *Dev Biol* 2007; **304**: 786-799.
12. Willet SG, Hale MA, Grapin-Botton A, *et al.* Dominant and context-specific control of endodermal organ allocation by Ptf1a. *Development* 2014; **141**: 4385-4394.
13. Kim TH, Shivdasani RA. Stomach development, stem cells and disease. *Development* 2016; **143**: 554-565.
14. McCracken KW, Wells JM. Mechanisms of embryonic stomach development. *Semin Cell Dev Biol* 2017; **66**: 36-42.
15. Willet SG, Mills JC. Stomach Organ and Cell Lineage Differentiation: from Embryogenesis to Adult Homeostasis. *Cell Mol Gastroenterol Hepatol* 2016; **2**: 546-559.
16. Offield MF, Jetton TL, Labosky PA, *et al.* PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 1996; **122**: 983-995.
17. Que J, Luo X, Schwartz RJ, *et al.* Multiple roles for Sox2 in the developing and adult mouse trachea. *Development* 2009; **136**: 1899-1907.
18. Arceci RJ, King AA, Simon MC, *et al.* Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol* 1993; **13**: 2235-2246.
19. Bossard P, Zaret KS. GATA transcription factors as potentiators of gut endoderm differentiation. *Development* 1998; **125**: 4909-4917.

20. Rojas A, De Val S, Heidt AB, *et al.* Gata4 expression in lateral mesoderm is downstream of BMP4 and is activated directly by Forkhead and GATA transcription factors through a distal enhancer element. *Development* 2005; **132**: 3405-3417.
21. Rojas A, Kong SW, Agarwal P, *et al.* GATA4 is a direct transcriptional activator of cyclin D2 and Cdk4 and is required for cardiomyocyte proliferation in anterior heart field-derived myocardium. *Mol Cell Biol* 2008; **28**: 5420-5431.
22. Rojas A, Schachterle W, Xu SM, *et al.* An endoderm-specific transcriptional enhancer from the mouse Gata4 gene requires GATA and homeodomain protein-binding sites for function in vivo. *Dev Dyn* 2009; **238**: 2588-2598.
23. Nemer G, Nemer M. Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. *Dev Biol* 2003; **254**: 131-148.
24. Jacobsen CM, Mannisto S, Porter-Tinge S, *et al.* GATA-4:FOG interactions regulate gastric epithelial development in the mouse. *Dev Dyn* 2005; **234**: 355-362.
25. Jacobsen CM, Narita N, Bielinska M, *et al.* Genetic mosaic analysis reveals that GATA-4 is required for proper differentiation of mouse gastric epithelium. *Dev Biol* 2002; **241**: 34-46.
26. Jonckheere N, Vincent A, Franquet-Ansart H, *et al.* GATA-4/-6 and HNF-1/-4 families of transcription factors control the transcriptional regulation of the murine Muc5ac mucin during stomach development and in epithelial cancer cells. *Biochim Biophys Acta* 2012; **1819**: 869-876.
27. Kuo CT, Morrisey EE, Anandappa R, *et al.* GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev* 1997; **11**: 1048-1060.
28. Molkenkin JD, Lin Q, Duncan SA, *et al.* Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev* 1997; **11**: 1061-1072.
29. Narita N, Bielinska M, Wilson DB. Wild-type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. *Dev Biol* 1997; **189**: 270-274.
30. Battle MA, Bondow BJ, Iverson MA, *et al.* GATA4 is essential for jejunal function in mice. *Gastroenterology* 2008; **135**: 1676-1686 e1671.
31. Xuan S, Sussel L. GATA4 and GATA6 regulate pancreatic endoderm identity through inhibition of hedgehog signaling. *Development* 2016; **143**: 780-786.
32. Shaw-Smith C, De Franco E, Lango Allen H, *et al.* GATA4 mutations are a cause of neonatal and childhood-onset diabetes. *Diabetes* 2014; **63**: 2888-2894.
33. Carrasco M, Delgado I, Soria B, *et al.* GATA4 and GATA6 control mouse pancreas organogenesis. *J Clin Invest* **122**: 3504-3515.
34. Xuan S, Borok MJ, Decker KJ, *et al.* Pancreas-specific deletion of mouse Gata4 and Gata6 causes pancreatic agenesis. *J Clin Invest* **122**: 3516-3528.
35. Watt AJ, Zhao R, Li J, *et al.* Development of the mammalian liver and ventral pancreas is dependent on GATA4. *BMC Dev Biol* 2007; **7**: 37.
36. Hingorani SR, Petricoin EF, Maitra A, *et al.* Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003; **4**: 437-450.
37. Srinivas S, Watanabe T, Lin CS, *et al.* Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* 2001; **1**: 4.

38. Kawaguchi Y, Cooper B, Gannon M, *et al.* The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat Genet* 2002; **32**: 128-134.
39. Delgado I, Carrasco M, Cano E, *et al.* GATA4 loss in the septum transversum mesenchyme promotes liver fibrosis in mice. *Hepatology* 2014; **59**: 2358-2370.
40. Munoz-Bravo JL, Hidalgo-Figueroa M, Pascual A, *et al.* GDNF is required for neural colonization of the pancreas. *Development* 2013; **140**: 3669-3679.
41. Kobayashi H, Spilde TL, Li Z, *et al.* Lectin as a marker for staining and purification of embryonic pancreatic epithelium. *Biochem Biophys Res Commun* 2002; **293**: 691-697.
42. Bosse T, Piaseckyj CM, Burghard E, *et al.* Gata4 is essential for the maintenance of jejunal-ileal identities in the adult mouse small intestine. *Mol Cell Biol* 2006; **26**: 9060-9070.
43. Thompson CA, Wojta K, Pulakanti K, *et al.* GATA4 Is Sufficient to Establish Jejunal Versus Ileal Identity in the Small Intestine. *Cell Mol Gastroenterol Hepatol* 2017; **3**: 422-446.
44. Raghoebir L, Bakker ER, Mills JC, *et al.* SOX2 redirects the developmental fate of the intestinal epithelium toward a premature gastric phenotype. *J Mol Cell Biol* 2012; **4**: 377-385.

## Figure Legends

### Figure 1. Abnormal morphology of the glandular stomach in *Gata4*-deficient mice.

Immunofluorescence analyses demonstrate overlapping expression of GATA4 and PDX1 in the antrum of wild-type embryos (A-C). Strong eYFP staining in the antrum (a) of *Pdx1-Cre; Rosa26RYFP* (*Pdx1-Cre;eYFP*) and *Gata4<sup>flox/flox</sup>; Pdx1-Cre; ROSA26ReYFP* (G4KO;*eYFP*) embryos at E17.5. A mosaic pattern for eYFP is found in the greater curvature of the corpus (c) (D, E). Higher magnification of the respective antral region are shown in F and G. Higher magnification of the respective corpus region are shown in H and I. Loss of *Gata4* in the antrum of *Gata4<sup>flox/flox</sup>; Pdx1-Cre* (G4KO) of E17.5 embryos (K) compared to Ctrl embryos (J). Strong GATA4 staining is observed in the corpus of E17.5 Ctrl embryos (L), whereas incomplete inactivation of *Gata4* is detected in the corpus of G4KO embryos (M). Scale bars: (A-G): 100  $\mu\text{m}$ ; (H-M): 25 $\mu\text{m}$ .

### Figure 2. Defects in morphology and cell differentiation in *Gata4*-deficient gastric epithelium.

Hematoxylin/Eosin staining of stomach tissue shows the antral epithelium of P1 *Gata4*-deficient mice (G4KO) is flattened and glands are not properly formed (B, D), in contrast to control (Ctrl) antrum epithelium in which the developing unit glands are easily observed (A, C). In the adult, the stomach of control mice displays well-organized gastric glands (E, G). G4KO antrum displays abnormal glandular morphology composed of epithelial cells with cornified appearance (F, H). Inset in H shows higher magnification of the cornified gastric epithelium.



Immunostaining analysis of the stomach of control (Ctrl) and *Gata4 KO* (G4KO) at P1 and adult stages for the parietal cell marker, HK<sup>+</sup>-ATPase (I-L), the pit cell marker MUC5AC (M-P) and G cell marker gastrin (Q-T) reveals defects in glandular cell differentiation in *Gata4KO* (G4KO) mice. Inset in S shows higher magnification of gastrin positive cells. Scale bars: (A, B, G, H, S, T): 100μm; (C, D, I, J, M, N, Q, R): 25μm; (E, F, K, L, O, P): 500μm.

### **Figure 3. GATA4 maintains stomach glandular cell identity**

Immunostaining for cytokeratin 14 revealed a clear junction between the forestomach and hindstomach in control (Ctrl) mice at both P1 (A, A') and adult stages (G, G'). *Gata4KO* mice exhibit upregulation of cytokeratin 14 expression in the corpus and antrum at P1 (B, B', B'') and at adult ages (H, H'). GATA4 is exclusively expressed in the columnar epithelium of P1 (C, C', E, E') and adult control stomach (I, I', K, K'). TRP63 protein is restricted to the forestomach in control mice at P1 (C, C') and in adult stages (I, I'). In *Gata4KO* mice, TRP63 immunostaining is observed in the antrum and corpus of neonate (D, D'') and adult (J, J') mice. *Sox2* is highly expressed in the forestomach of control neonate (E, E') and adult (K, K') mice. An upregulation of *Sox2* expression is observed in the antrum and corpus of *Gata4KO* mice at P1 (F, F'') and adult ages (L, L'). Note that cells that escape from Cre recombination in the corpus of *Gata4KO* mice are positive for GATA4 and negative for TRP63 (D') and SOX2 (F'). Counterstaining with DAPI (blue) was performed to visualize nuclei in immunofluorescence analyses. fs: forestomach; c: corpus, a: antrum. A' through F'' are high magnification pictures from A through F, respectively. Scale bars: (A-F, G-L): 500μm; (A'-F'', G'-L'): 100μm.

**Figure 4: Ectopic formation of pancreatic tissue in the distal stomach of *Gata4*-deficient mice.** Histological and immunofluorescence analyses of the stomach of control (Ctrl) and *Gata4* knockout (G4KO) E17.5 embryos (A-F), P1 neonates (G-I) and adult mice (J-R). Embryonic G4KO stomach tissue displays invagination of the antrum epithelium into the subserosa layer forming a duct-like structure (D), as observed by Hematoxylin/Eosin staining. Normal stomach morphology in control embryos (A). Hematoxylin/Eosin staining reveals well-distinguishable pancreatic tissue in the subserosa of *Gata4KO* stomach at P1 (G) and adult stages (J, M). eYFP-labelled cells in the ectopic pancreas of adult *Gata4<sup>fllox/fllox</sup>; Pdx1-Cre ROSA26R-eYFP* mice are continuous with the stomach epithelium and penetrate the submucosal and muscular layers of the antrum, marked by smooth  $\alpha$ -actin ( $\alpha$ -SMA) immunoreactivity (E, H, K). Ectopic pancreas of *Gata4KO* contains well-differentiated acinar cells that express amylase (F, I, N). Ectopic pancreatic epithelium, marked by Ecadherin, show expression of the acinar marker PTF1A (O) . At adult stages, small clusters composed of endocrine insulin- and glucagon-expressing cells (P) are observed. Insulin-expressing cells express MAFA (Q). Insulin-expressing cells, marked by PDX1 immunoreactivity, also express the glucose transporter GLUT2 (R) . Counterstaining with DAPI was performed to visualize nuclei (blue) in immunofluorescence analyses. Scale bars: (A-N, P-R):100 $\mu$ m. (O): 25 $\mu$ m.

**Figure 5: Pancreatic marker expression in the glandular stomach of *Gata4KO* embryos.** Immunofluorescence analyses reveal the presence of insulin-positive cells in the developing glandular stomach (B) and proximal duodenum (D) of E17.5 *Gata4* knockout (G4KO) embryos. No insulin positive cells are found in stomach or

duodenum epithelium of E17.5 control embryos (A, C). Areas of the *G4KO* glandular epithelium invaginating into the subserosa layer and discontinuing the smooth muscle layer exhibited upregulation of the ductal pancreatic marker Hnf1 $\beta$  (F), in parallel with increased reactivity for the ductal marker DBA lectin (H). No immunostaining for HNF1 $\beta$  and DBA lectin is observed in control stomach (E, G). Counterstaining with DAPI was performed to visualize nuclei (blue). Arrowheads in B and D indicate insulin-producing cells. Scale bars: (A-F): 100 $\mu$ m; (G, H): 25 $\mu$ m.

**Figure 6: *GATA4* expression in human heterotopic pancreas.**

Hematoxylin/eosin and immunofluorescence analyses of human gastric biopsies of control individuals (A-E) and patients with heterotopic pancreas (F-O). Eight (out of 24) biopsies of heterotopic pancreas display *GATA4* protein levels in the ectopic pancreas (G) and in the glandular stomach (I) similar to those observed in normal pancreas (B) and in stomach of healthy patients (D). However, the remaining 16 biopsies exhibit a dramatic decrease in the accumulation of *GATA4* protein in both the ectopic pancreas (L) and proximal duodenum (N). Of note, all patients analyzed show similar (and robust) levels of amylase protein in the pancreatic tissue (E, J, O). Scale bars: (A-D, F-I, K-N): 100 $\mu$ m; (E, J, O): 25 $\mu$ m.

**Supplemental figure legends**

**Supplemental Figure 1:** Hematoxylin/Eosin staining of stomach tissue for control (Ctrl) and *Gata4* knockout (G4KO) embryos at E13.5 (A, B), E15.5 (C, D) and E17.5 (E, F). No morphological anomalies are observed in the stomach epithelium of E13.5 and E15.5 G4KO embryos, compared to Ctrl embryos (n=5 embryos analyzed for each stage and group). At E17.5, developing gastric units are well formed in control embryos

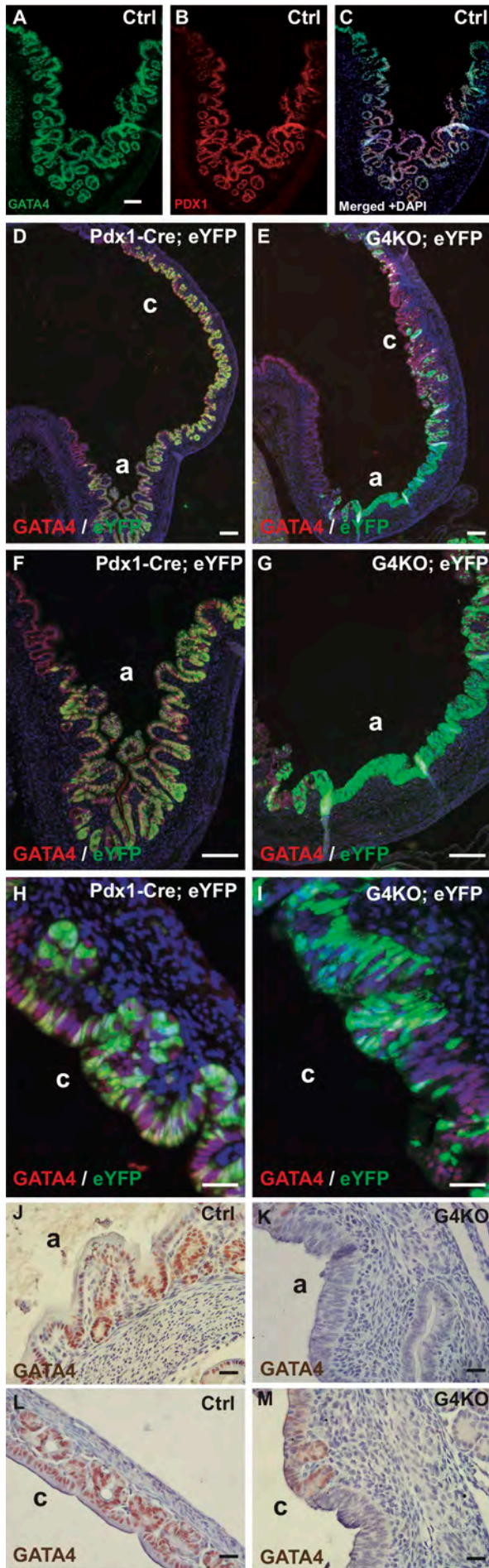
(E) while G4KO stomach lacks gastric glands and the epithelium is flattened (F). Immunohistochemistry analyses revealed robust expression of parietal (HK<sup>+</sup>-ATPase) and G cell (Gastrin) markers in control stomach (G and I, respectively). By contrast, the G4KO embryonic stomach fails to express any of these gastric cell markers (H, J). Scale bars: 25µm

**Supplemental Figure 2: Up-regulation of *Sonic hedgehog* expression in *Gata4KO* antrum and normal *Ngn3* and *Hes1* expression in the pancreas and stomach of**

***Gata4KO* embryos.** Immunofluorescence analysis using Sonic hedgehog (SHH) antibody (goat anti-Shh (R&D Systems, AF445) show strong GATA4 expression but low SHH expression in E17.5 control antrum (Ctrl) (A-C). By contrast increased SHH expression is observed in the antrum of *Gata4 KO* (G4KO) embryos (D-F). Of note, SHH is absent in the ectopic pancreas (ep). Dissected pancreata and stomach from three E13.5 or E15.5 embryos of control (Ctrl) or *Gata4KO* (G4KO) embryos were pooled to obtain RNA. Expression of *Ngn3* and *Hes1* was examined by quantitative RT-PCR in three different samples of stomach (G) and dorsal pancreas (H) at E13.5 and E15.5 control (Ctrl) and *Gata4* knockout (G4KO) embryos (G). Total RNA isolation and qPCR was performed as previously described (Carrasco et al., Journal of Clinical Investigation, 2012, 112, 3504-3515), using the following Taqman probes (Applied Biosystems): *Ngn3*: Mm\_00437606; *Hes1*: Mm\_01342805\_m1;  $\beta$ -actin: Mm02619580\_g1. Gene expression data are normalized to  $\beta$ -actin mRNA levels and presented as mean  $\pm$  SEM. Immunofluorescence analysis of E-cadherin and smooth muscle  $\alpha$  actin (I) shows normal morphology of the dorsal pancreatic epithelium of E13.5 *G4KO* embryos without contact with the glandular stomach epithelium, similar

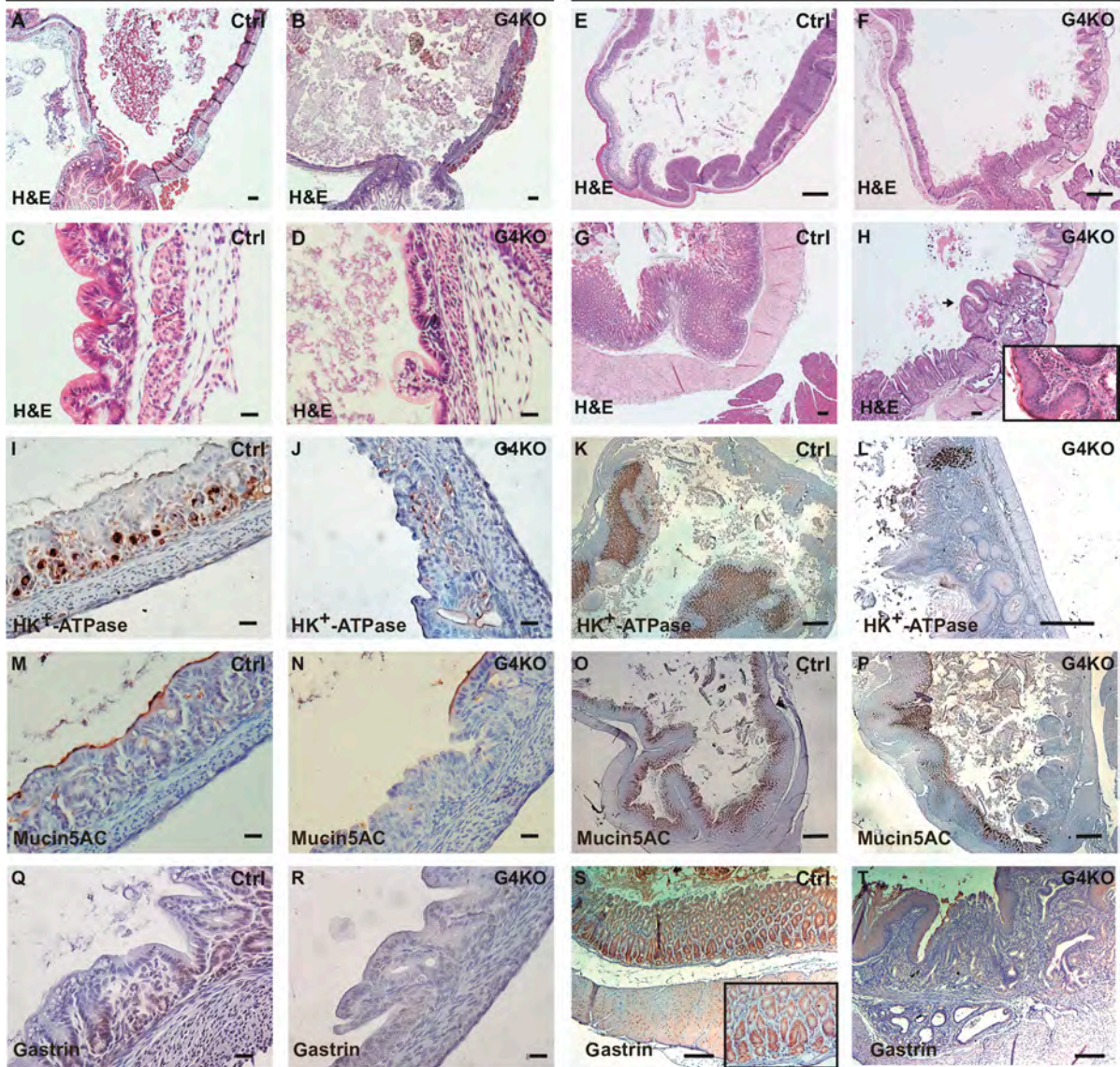
to what is observed in E13.5 control embryos (J). Counterstaining with DAPI was performed to visualize nuclei (blue). Scale bars: (A-F): 25 $\mu$ m; (H, I) 100 $\mu$ m.

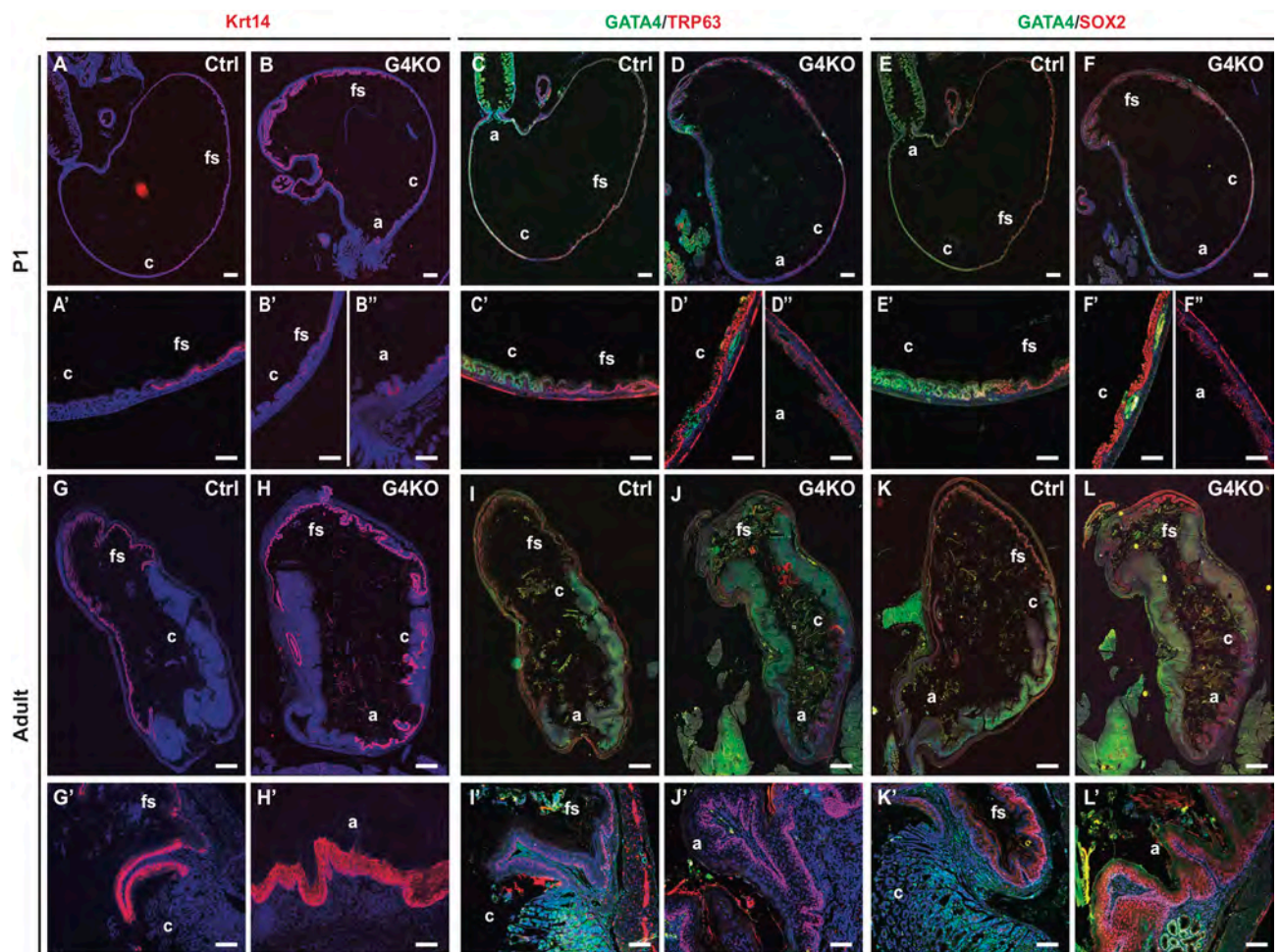
**Supplemental Figure 3: Normal glandular stomach morphology in *Gata4*<sup>flox/flox</sup>; *Ptfla-Cre* mice.** Hematoxylin/eosin staining reveals similar morphology of the glandular stomach in control (A) and *Gata4*<sup>flox/flox</sup>; *Ptfla-Cre* mutant mice (B) at P1. Immunohistochemical analyses show strong GATA4 (C, D) and HK<sup>+</sup>-ATPase (E, F) expression in the glandular stomach of both control and *Gata4*<sup>flox/flox</sup>; *Ptfla-Cre* P1 neonatal mice.



P1

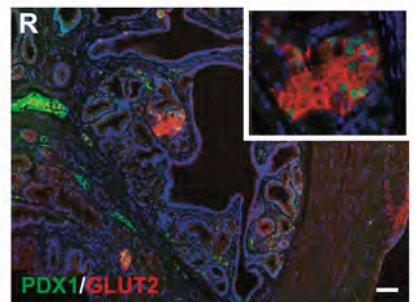
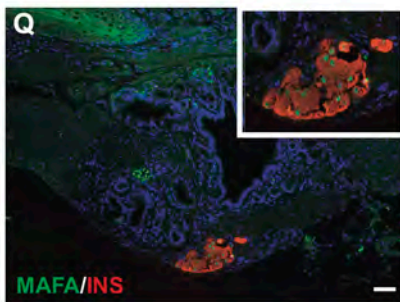
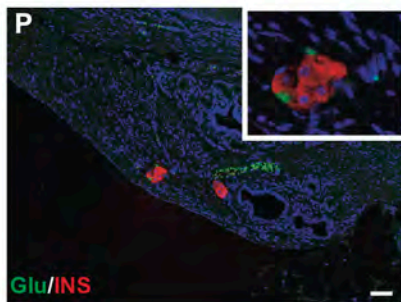
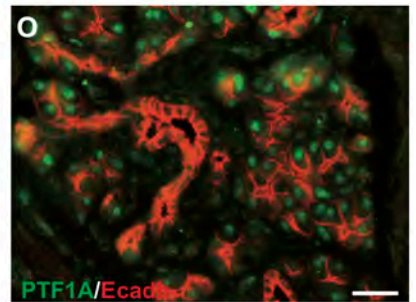
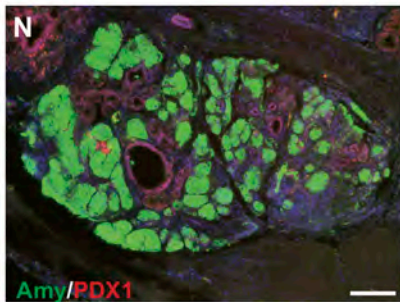
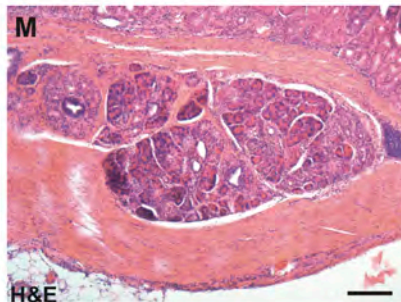
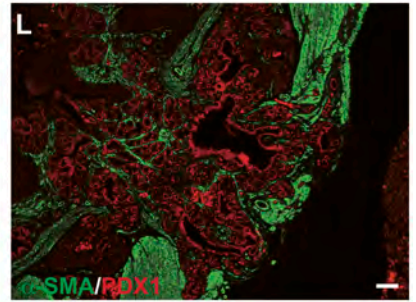
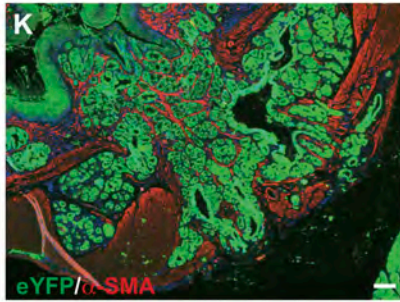
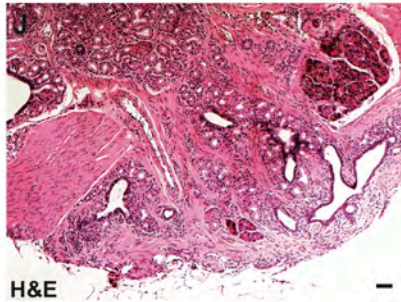
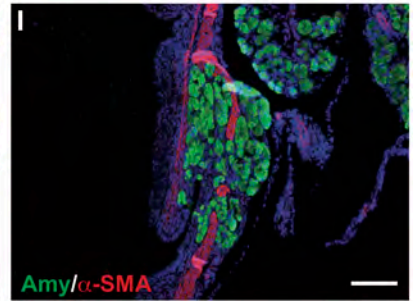
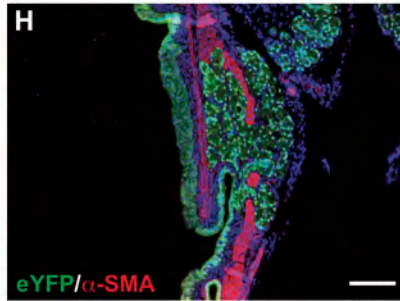
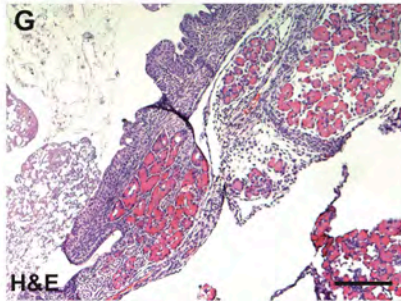
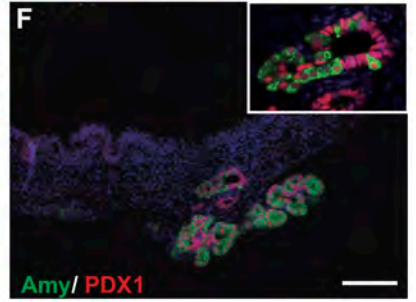
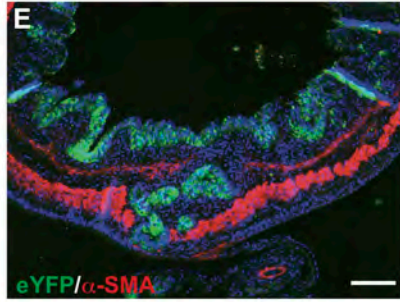
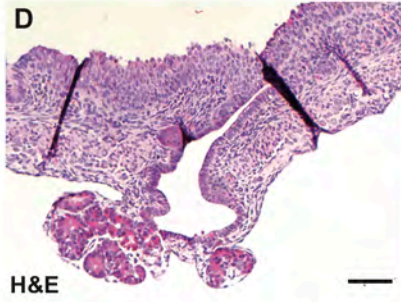
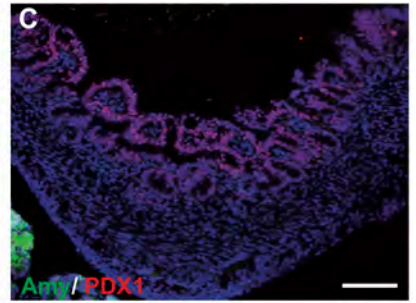
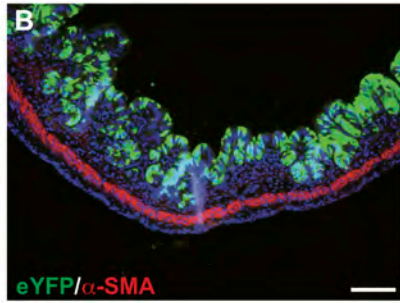
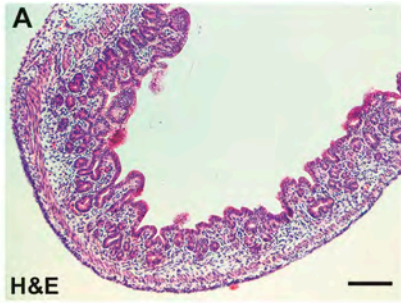
Adult



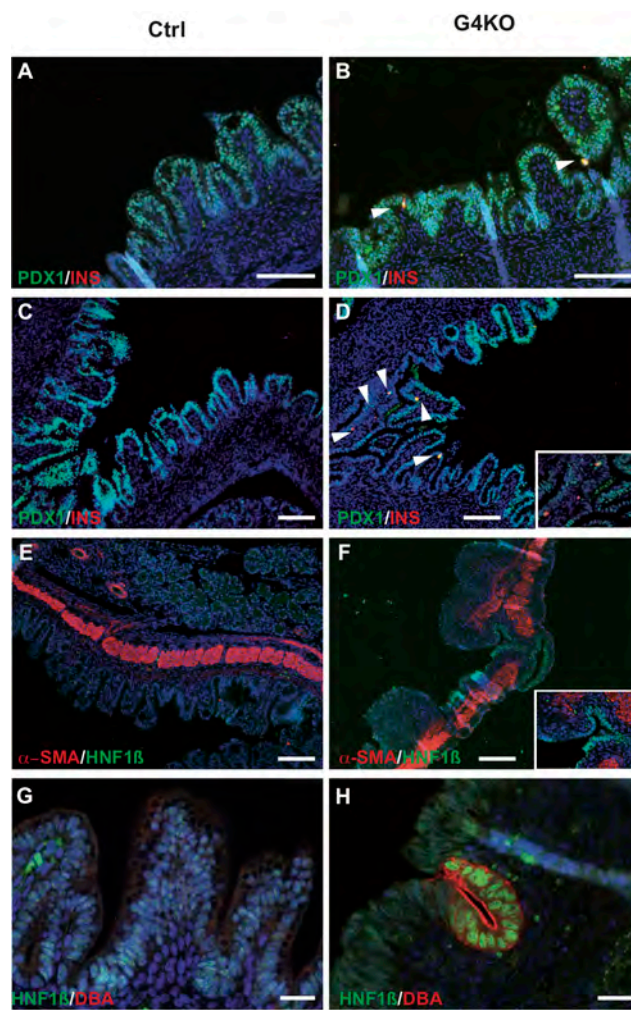


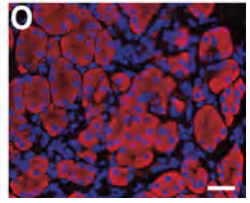
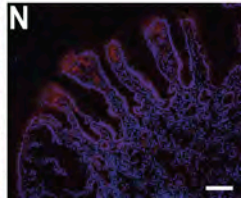
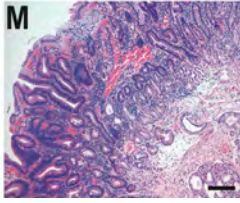
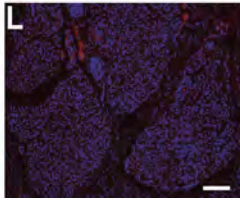
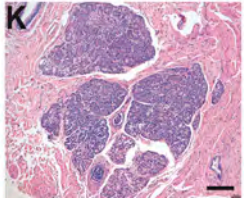
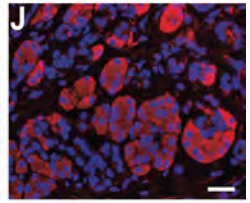
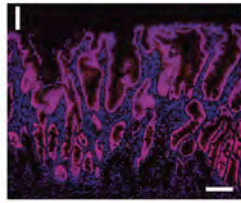
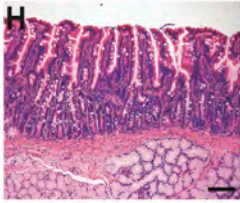
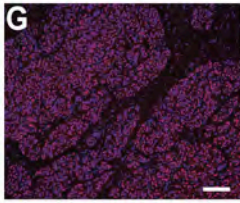
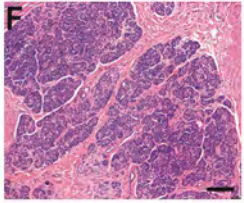
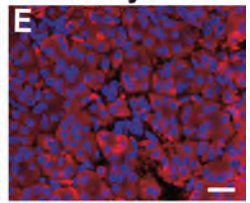
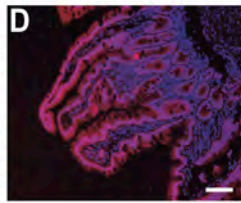
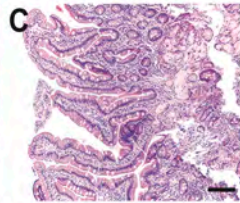
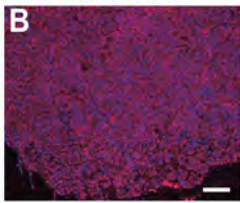
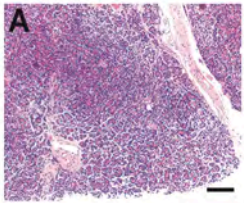
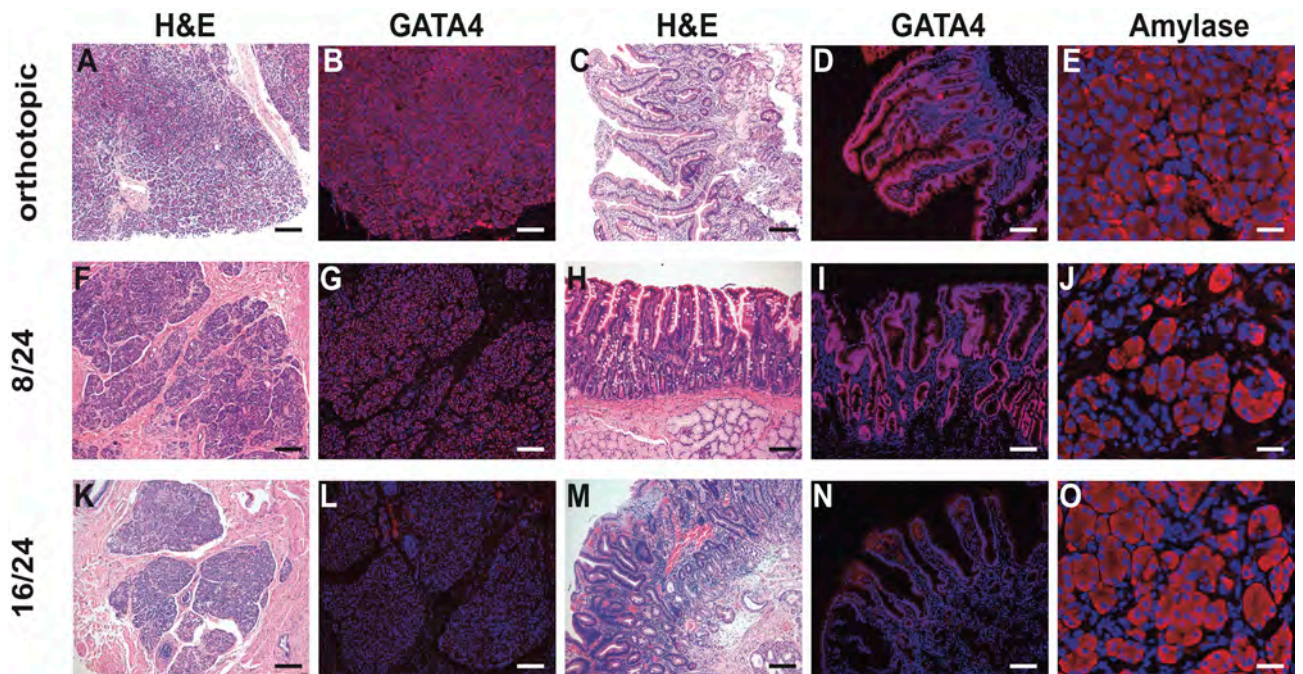


Ctrl



G4KO





orthotopic

8/24

16/24