



## Arx is required for normal enteroendocrine cell development in mice and humans

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### ABSTRACT

Enteroendocrine cells of the gastrointestinal (GI) tract play a central role in metabolism, digestion, satiety and lipid absorption, yet their development remains poorly understood. Here we show that *Arx*, a homeodomain-containing transcription factor, is required for the normal development of mouse and human enteroendocrine cells. *Arx* expression is detected in a subset of Neurogenin3 (*Ngn3*)-positive endocrine progenitors and is also found in a subset of hormone-producing cells. In mice, removal of *Arx* from the developing endoderm results in a decrease of enteroendocrine cell types including gastrin-, glucagon/GLP-1-, CCK-, secretin-producing cell populations and an increase of somatostatin-expressing cells. This phenotype is also observed in mice with endocrine-progenitor-specific *Arx* ablation suggesting that *Arx* is required in the progenitor for enteroendocrine cell development. In addition, depletion of human *ARX* in developing human intestinal tissue results in a profound deficit in expression of the enteroendocrine cell markers CCK, secretin and glucagon while expression of a pan-intestinal epithelial marker, *CDX2*, and other non-endocrine markers remained unchanged. Taken together, our findings uncover a novel and conserved role of *Arx* in mammalian endocrine cell development and provide a potential cause for the chronic diarrhea seen in both humans and mice carrying *Arx* mutations.

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### Introduction

The mammalian gastrointestinal (GI) tract is lined with a single layer of epithelial cells that undergo continuous renewal throughout life. In the stomach and intestine, the self-renewing stem cells are the source of all epithelial cell types and are located in the neck of the gastric glands and in the crypts of intestine (Mills and Shivdasani, 2011; Simons and Clevers, 2011). Epithelial cell types in the stomach include mucin-secreting cells, pepsinogen-producing chief cells and acid-secreting parietal cells, whereas the intestine has absorptive enterocytes, mucus-secreting goblet cells, lysozyme-producing Paneth cells and secretory tuft cells (Gerbe et al., 2009, 2011; May and Kaestner, 2010). In addition, enteroendocrine cells secreting at least 15 different hormones are found interspersed along the epithelium of the digestive tract (May and Kaestner, 2010; Rindi et al., 2004).

Representing one of the complex endocrine systems in the body, the enteroendocrine system plays a central role in metabolism, digestion, satiety and lipid absorption (Beglinger and Degen, 2004; Drucker, 2007a, 2007b; Gershon, 1968; Mellitzer and Gradwohl, 2011; Murphy and Bloom, 2006; Murphy et al., 2006; Rehfeld, 2004a, 2004b). The importance of these cells is demonstrated by studies in which patients with malabsorptive diarrhea were found to have mutations in the *NEUROGENIN3* gene, which resulted in undetectable enteroendocrine cells (Rubio-Cabezas et al., 2011; Wang et al., 2006). Despite the unequivocal importance of enteroendocrine cells in the regulation of diverse biological processes, there is a deficiency in our understanding of how the specific enteroendocrine subtypes are generated during GI development in mice and humans.

The mechanism by which enteroendocrine cells regulate the above biological processes is via an array of secreted hormones. In the intestine, cholecystokinin (CCK) is known to promote digestive enzyme release and gallbladder contraction, secretin is important to stimulate pancreatic enzyme release and biliary bicarbonate production, and glucagon-like peptide-1 (GLP-1) is involved in potentiating glucose-dependent insulin secretion and inhibits gastric emptying (Baggio and Drucker, 2007; Lloyd, 1994; Murphy and Bloom, 2006; Rehfeld, 2004b; Wren, 2008). In the stomach, hormones such as

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gastrin and somatostatin are important to stimulate and inhibit gastric acid release, respectively, while ghrelin is critical to control appetite (Schubert, 2010; Wren and Bloom, 2007; Wren et al., 2001).

Several transcriptional regulators have been shown in mice to regulate development of the enteroendocrine cells (May and Kaestner, 2010). While some factors are required for general endocrine cell differentiation (i.e. Neurogenin3 (Ngn3), Math1), others are only necessary to specify enteroendocrine subtypes. Ngn3, a proendocrine transcription factor, is necessary for the formation of enteroendocrine progenitors in the GI tract, as *Ngn3*-deficient mice display a complete and a partial loss of enteroendocrine cells in the intestine and stomach, respectively, similar to what has been reported in humans (Jenny et al., 2002; Lee et al., 2002; Mellitzer et al., 2010; Wang et al., 2006). In addition, *Math1* null mice show a complete deficiency in all secretory lineages including enteroendocrine cells, Paneth cells, and goblet cells in the intestine (Yang et al., 2001). In contrast, homeodomain transcription factors such as Pax4, Pax6, Nkx6.1, Nkx2.2, Nkx6.3, and Pdx1 are involved in the differentiation of specific enteroendocrine cell types (Choi et al., 2008; Guz et al., 1995; Jonsson et al., 1994, 1995; Larsson et al., 1996, 1998; Offield et al., 1996). Taken together, these studies, largely in mice, demonstrate the importance of transcriptional regulation in enteroendocrine cell differentiation in the GI tract and provide the basic framework for our current understanding of how these specialized cells are formed during development. However, the mechanism of cell fate determination of such a diverse group of hormone producing cells from a single *Ngn3*<sup>+</sup> progenitor population (Schonhoff et al., 2004) is largely unknown.

Here, we report a previously unappreciated role for *Arx*, a gene encoding a *prd*-homeodomain-containing transcription factor, during enteroendocrine cell development in mice and humans. We find that *Arx* is expressed in a subset of *Ngn3*<sup>+</sup>-endocrine progenitors and a subset of mature hormone producing-cells in the developing GI tract and that *Arx* is required for development of enteroendocrine population. Furthermore, the requirement of *ARX* was further examined in human proximal intestinal organoids generated from human embryonic stem (hES) cells (Spence et al., 2011), where *ARX* knock-down resulted in a significant decrease in the enteroendocrine cell markers CCK, secretin and glucagon.

Taken together, this study is the first to establish *Arx* as a novel and important factor during enteroendocrine cell differentiation in both mouse and human. Analysis performed in mice further shows that *Arx* functions in the *Ngn3*<sup>+</sup> endocrine progenitors to promote gastrin, glucagon/GLP-1, CCK and secretin lineages, while simultaneously repressing the somatostatin cell lineage in the GI tract. Given that both *Arx*-deficient mice and patients with *ARX* mutations suffer chronic diarrhea (Itoh et al., 2010; Kato and Dobyns, 2005), these findings also provide strong evidence that the primary cause of diarrhea in these patients is likely due to alterations of specific enteroendocrine cell populations.

## Methods

### Mice and tissue preparation

CD1 wild-type mice were used for characterizing *Arx* expression. *Arx* is X-linked and the derivation of the floxed conditional allele (*Arx*<sup>L/L</sup>), *Foxa3-Cre*, *Ngn3-Cre* and *Villin-Cre* mouse lines has been reported previously (el Marjou et al., 2004; Fulp et al., 2008; Lee et al., 2005; Schonhoff et al., 2004). These mice were maintained on a mixed background (CD1, 129 and C57BL/6) and were cared and handled according to the Children's Hospital of Philadelphia's Institutional Animal Care and Use Committee approved protocol. Timed pregnancies were determined based on the appearance of the vaginal plugs and were considered embryonic day (E) 0.5.

Primers for genotyping alleles used in this study were described previously (Fulp et al., 2008; Lee et al., 2005; Schonhoff et al., 2004).

### Real-time PCR analysis

All tissue dissections were performed in cold 1X PBS, and the tail snips were used for genotyping. Total RNA was extracted in TRIZOL (Invitrogen) using the RNA Easy kit (Qiagen). Oligo-dT, superscript plus other required reagents were used to synthesize cDNA. PCR reactions were set up using the Brilliant SYBR Green PCR Master Mix in the Stratagene Mx3005P Real-time PCR machine. All reactions were performed in triplicate with reference dye normalization. Primer sequences are available upon request.

### Immunohistochemistry and histology

Tissues were fixed in 4% paraformaldehyde overnight at 4 °C and embedded in paraffin or optimal cutting temperature (OCT) freezing medium, and 8 μm sections collected. Slides were subjected to microwave antigen retrieval in 10 mmol/L sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> in PBS for 15 min, and sections blocked with CAS-Block reagent (Invitrogen). The sections were incubated with primary antibodies overnight at 4 °C and appropriate secondary antibodies for 2 h at room temperature. Immunohistochemical detection was performed with the VECTASTAIN ABC kit (Vector Laboratories) and diaminobenzidine tetrahydrochloride (DAB) as the substrate. The primary antibodies used were: Glucagon (1:3000; Millipore), Somatostatin (1:3000; Santa Cruz), *Arx* (1:500; gift from Dr. Kanako Miyabayashi at Kyushu University), *Pdx1* (1:200; Santa Cruz), Ghrelin (1:200; Santa Cruz), *Isl-1* (1:50; Developmental Studies Hybridoma Bank 39.4D5 and 40.2D6), Gastrin (1:200; Santa Cruz), 5-hydroxytryptamine: 5-HT (Serotonin) (1:50,000; ImmunoStar), Chromogranin A (1:3000; DiaSorin), Neurogenin 3 (1:500; Developmental Studies Hybridoma Bank), GLP-1 (1:500; Abcam), CCK (1:100; Santa Cruz), DCAMKL1 (1:100; Abcam), *Muc2* (1:750; Santa Cruz), Lysozyme (1:3000; Dako), *Cdx2* (1:500; Biogenex). Sections were stained with Hematoxylin and eosin (H&E), Alcian blue, and Oil-Red-O according to the standard protocols. Oil-Red-O staining was performed using frozen sections.

### Hormone cell quantification

The hormone-positive cells from antrum and corpus of the stomach were counted, combined and normalized to the total epithelial area of the same or adjacent section for the analyses in *Arx*<sup>Endoderm</sup> and control mice. Separate regions (antrum and corpus) of the stomach were counted and normalized to the respective epithelial areas of the same or adjacent section for the analyses in *Arx*<sup>Endocrine</sup> and control mice. Hormone-positive cells from different regions of the intestine were counted and normalized to the respective epithelial area of the same or adjacent section. Epithelial area was measured with Aperio Image Analysis System.

### Blood glucose measurement

Blood glucose was measured using an automatic glucometer (One Touch Ultra; LifeScan, Milpitas, CA).

### hES-derived intestinal organoid culture

The human ES cell line WA09 was obtained from WiCell and maintained in feeder-free conditions on Matrigel (BD Biosciences) in mTesR1 medium (Stem Cell Technologies). Stable shRNA knock-down lines were generated by lentiviral-mediated transduction of single-cell suspensions with MISSION shRNA clones from Sigma-Aldrich (*ARX* shRNA: TRCN000016333; non-targeting control

shRNA: SHC002). After transduction, stable lines were selected with puromycin and maintained under selection throughout differentiation protocols. Human intestinal organoids were generated as previously described (Spence et al., 2011). Briefly, ES cells were plated in a 24-well plate and grown to near-confluency. To generate definitive endoderm, cells were treated with Activin A (100 ng/ml; R&D Systems) for 3 days in RPMI 1640 medium (Invitrogen) in the presence of 0%, 0.2%, and 2% dFBS (Thermo Scientific) on days 1, 2, and 3, respectively. Endoderm cells were then cultured in DMEM/F12 medium (Invitrogen) in the presence of 2% dFBS, Wnt3a (500 ng/ml; R&D Systems), and FGF4 (500 ng/ml; R&D Systems) for 3–4 days. Floating spheroids generated from the hindgut cultures were embedded in Matrigel (BD Biosciences) and cultured in DMEM/F12 medium supplemented with 10  $\mu$ M HEPES, N2 Supplement (R&D Systems), B27 Supplement (Invitrogen), R-Spondin (500 ng/ml; R&D systems), Noggin (100 ng/ml; R&D Systems), and EGF (50 ng/ml; R&D Systems). Human intestinal organoids were passaged into fresh Matrigel every 14 days.

## Results

### *Arx* is expressed in a subset of enteroendocrine cells in the GI epithelium

Immunostaining analysis was performed to characterize the temporal and spatial expression domains of *Arx* in the developing stomach and intestine of mice. While *Arx* expression was strong in the pancreas at embryonic day (E)13.5, its expression was not detectable in the stomach and intestine at this stage (data not shown). A day later, *Arx* expression was detected at a low frequency in the duodenum, but not in the stomach (Fig. 1a and data not shown). At E15.5, the GI epithelium begins to differentiate and form gastric units and nascent villi and shortly after this (~E17.5), *Arx*-expressing cells can be found along the crypt-villus axis in the entire developing digestive tract (Fig. 1b–f). We assessed whether *Arx* expression was restricted to enteroendocrine cells using chromogranin A (ChrA), an endocrine cell marker, in postnatal day (P)3 stomach and duodenum, and found that *Arx* expression was restricted to a subset of the ChrA-positive cells (Fig. 1g and h). To evaluate which enteroendocrine subtypes express *Arx*, we analyzed *Arx* expression in specific gastrointestinal hormone-expressing cells. In the P3 stomach, *Arx* expression was localized to gastrin-, glucagon-, serotonin-, somatostatin-, and ghrelin-expressing cells (Fig. 1i–m). In the duodenum, *Arx* expression was found in enteroendocrine subpopulations including cholecystokinin- (CCK), ghrelin-, glucagon-, GLP-1-, and serotonin-positive cells (Fig. 1n–r). Interestingly, we did not detect colocalization of *Arx* with somatostatin in the duodenum (Fig. 1s). Lastly, *Arx* expression was not detected in the intestinal Paneth, tuft or goblet cells at all ages examined (Fig. 1t–w and data not shown). Taken together, these results indicate that *Arx* is restricted to a specific subset of enteroendocrine cells.

### *Arx* expression is found in subsets of *Pdx1*<sup>+</sup>, *Ngn3*<sup>+</sup> and *Isl-1*<sup>+</sup> cells

To investigate when *Arx* might function during enteroendocrine development, we compared *Arx* expression with key transcriptional regulators (*Pdx1*, *Ngn3*, or *Isl-1*) that have been associated with specific developmental stages of enteroendocrine cell differentiation (Das and May, 2011; Guz et al., 1995; Jenny et al., 2002; Jonsson et al., 1994, 1995; Larsson et al., 1995; Lee et al., 2002; Mellitzer et al., 2010; Offield et al., 1996). At E17.5, very few *Arx*-positive cells co-expressing *Pdx1* or *Ngn3* were found in the stomach and duodenum (Supple. Fig. 1a–i and data not shown). In addition, a few cells co-expressing *Arx* and *Isl-1* were detected in the duodenum (Supple. Fig. 1j–l). As *Ngn3* marks endocrine progenitors and *Isl-1* labels differentiated endocrine cells (Das and May, 2011; Jenny et al., 2002; Lee et al., 2002; Schonhoff et al., 2004), these findings

suggest that *Arx* functions downstream of *Ngn3* in the subset of undifferentiated endocrine progenitors and/or in the differentiated endocrine cells of the GI epithelium.

### *Endoderm-specific Arx*-deficient mice exhibit growth retardation and chronic diarrhea

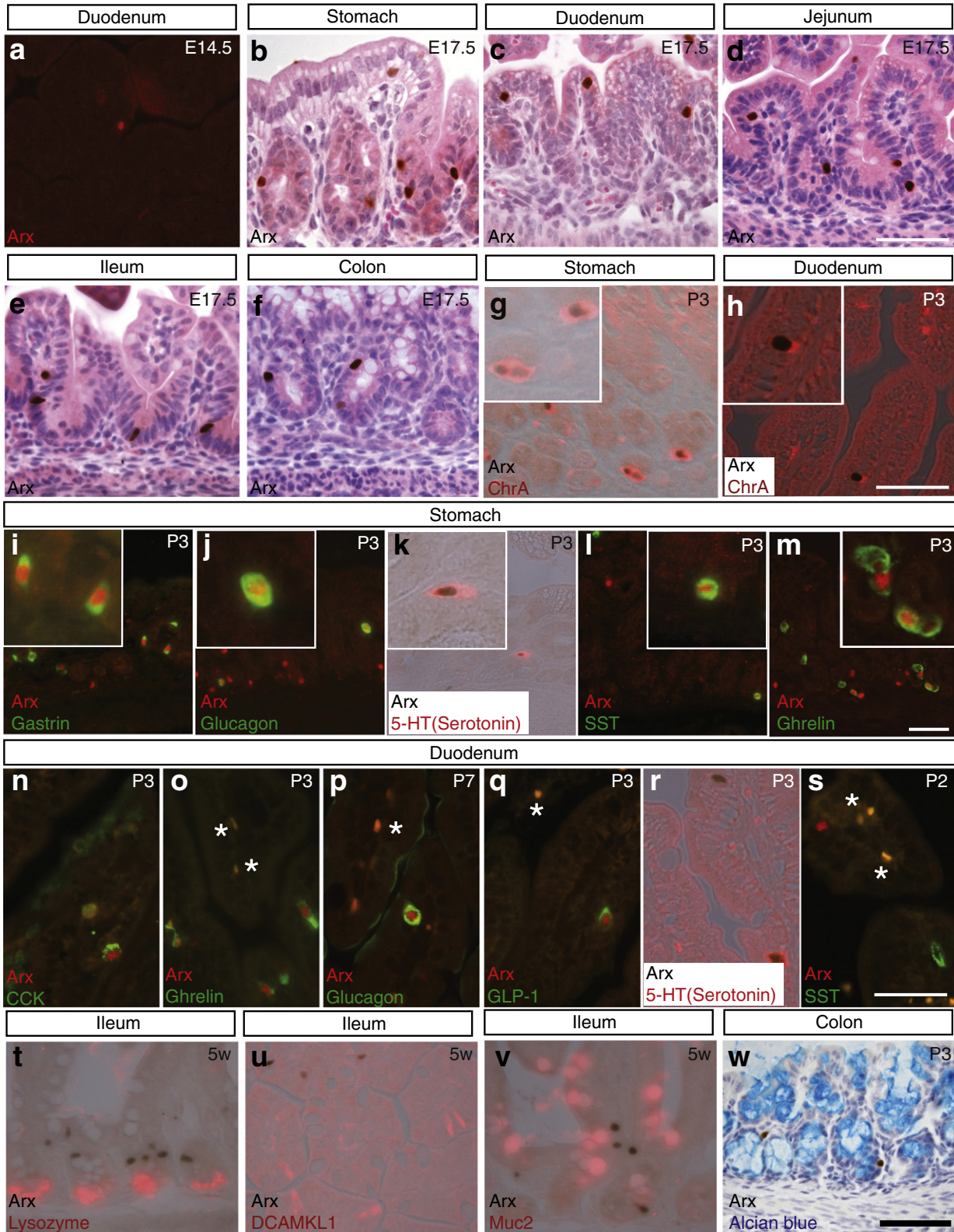
To examine the functional consequences of *Arx* deficiency in the epithelium of the GI tract, including stomach and intestine, we generated mice with *Arx* deletion in the developing endoderm (*Foxa3-Cre*; *Arx*<sup>L/L</sup> or *Foxa3-Cre*; *Arx*<sup>L/Y</sup>; referred to as *Arx*<sup>Endoderm</sup> mutant hereafter) by intercrossing *Foxa3-Cre* mice to *Arx*-floxed mice (Fulp et al., 2008; Lee et al., 2005). We assessed the efficiency of *Arx* removal in the developing stomach and intestine at the onset of enteroendocrine cell differentiation at E17.5. Compared to the control mice, *Arx*<sup>Endoderm</sup> mutants lacked all *Arx*-expressing cells at E17.5 in the stomach and duodenum (Fig. 2a–d and data not shown). Mutants were born at the expected Mendelian ratio and were outwardly indistinguishable from their control siblings at birth. However, in the next few days, *Arx*<sup>Endoderm</sup> mutants fell behind their control littermates in growth (Fig. 2e–f). By the end of the first week, all *Arx*<sup>Endoderm</sup> mutant animals had severe diarrhea and half of these mice died, the other half of the mutants survived past weaning and appeared grossly normal albeit reduced in size, and continued to have diarrhea (Fig. 2g). Early lethality was not due to lack of feeding as all mutant mice had milk in their stomach (Fig. 2h). Although the digestive tract was smaller and shorter in the *Arx*<sup>Endoderm</sup> mutants compared to the control littermates, these reductions were proportional to the length and body weight of the *Arx*<sup>Endoderm</sup> mutant mice (Fig. 2h and data not shown). In our macroscopic analyses of the *Arx*<sup>Endoderm</sup> mutants, we observed dilation in the ileum (arrows; Fig. 2h). Lastly, P3 *Arx*<sup>Endoderm</sup> mutant mice also displayed relative hypoglycemia (Fig. 2i), which can result from malnourishment due to chronic diarrhea (as discussed below) and/or due to islet glucagon cell deficiency in the pancreas (Collombat et al., 2003).

### Abnormal architecture observed in the ileal epithelium of *Arx*<sup>Endoderm</sup> mutant mice

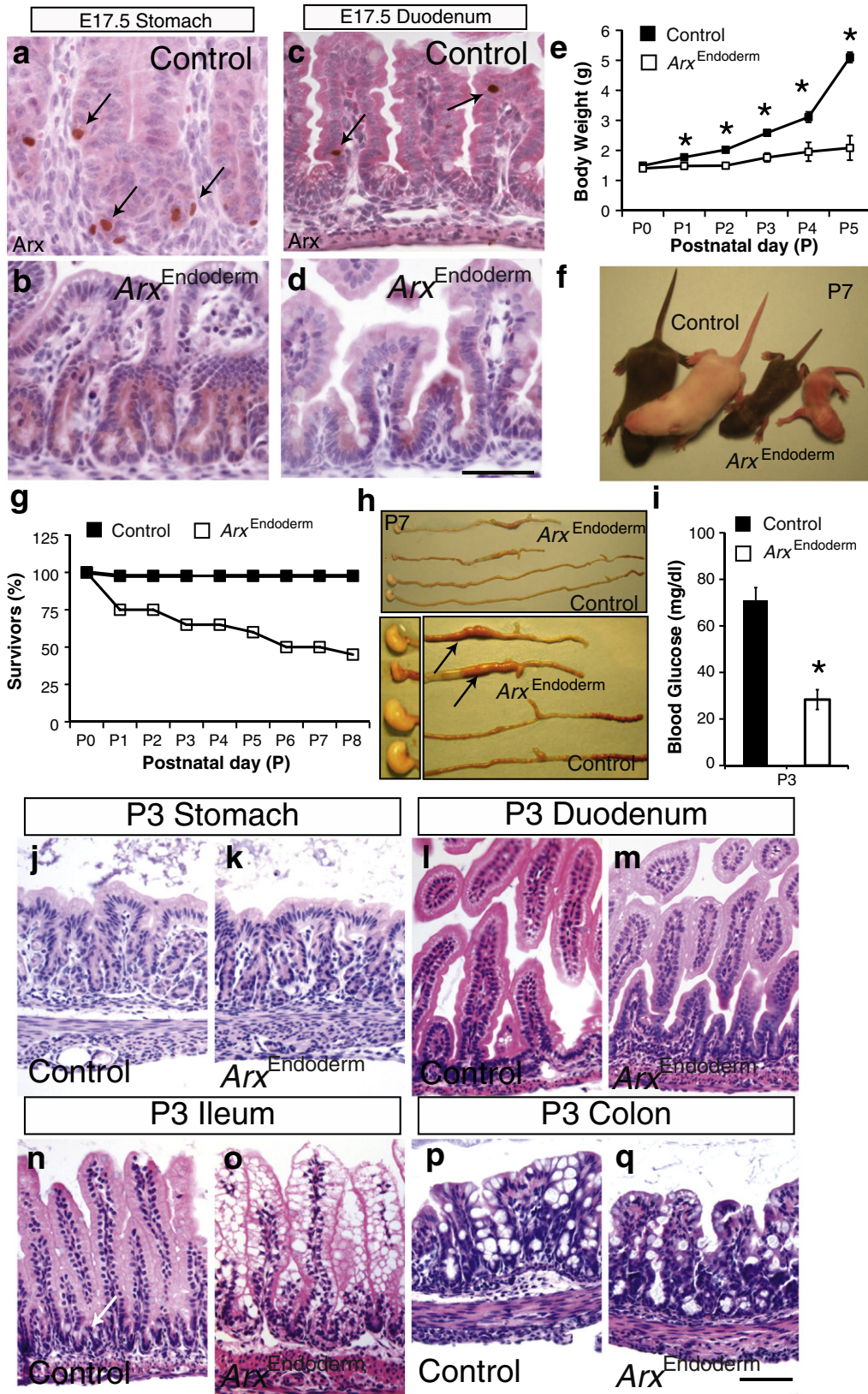
The impact of *Arx* removal on the overall histology of the GI epithelium was next assessed. Hematoxylin and eosin stained tissue sections from the control and *Arx*<sup>Endoderm</sup> mutant mice revealed normal architecture of the stomach, duodenum and colon; however, in the ileum we observed abnormal vacuoles in the enterocytes of *Arx*<sup>Endoderm</sup> mutant mice (Fig. 2j–q). Since *Arx* expression is not restricted to the ileum or found in enterocytes (data not shown), the histological changes seen in the ileum are likely secondary to the loss of *Arx* in enteroendocrine cell populations (see below for further discussion).

### Gut hormones critical for digestion and motility are affected in *Arx*<sup>Endoderm</sup> mutant mice

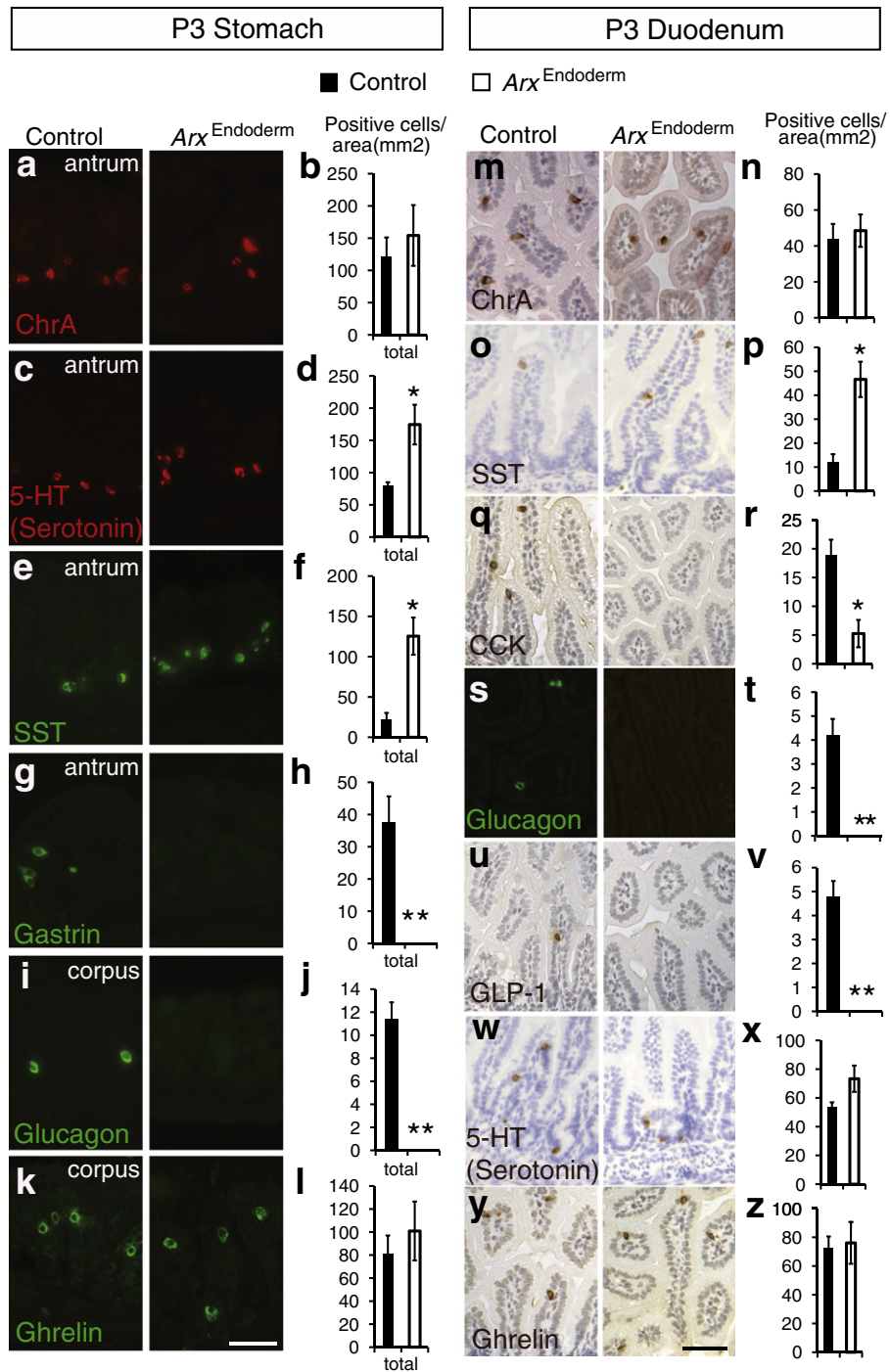
As *Arx* is expressed in developing and postnatal enteroendocrine cells (Fig. 1 and Supple. Fig. 1), we investigated the impact of *Arx* deletion on formation of enteroendocrine cells using the pan-endocrine marker ChrA. While the total number of ChrA-positive enteroendocrine cells remained unchanged, the proportion of serotonin- and somatostatin-expressing cells was increased, and the frequency of gastrin- and glucagon-expressing cells was reduced in the P3 *Arx*<sup>Endoderm</sup> mutant stomach (Fig. 3a–j). Although *Arx* expression was detected in a subset of ghrelin-expressing cells (Fig. 1m), we did not detect a difference in the number of this cell type (Fig. 3k–l). Similar analyses were conducted in the P3 duodenum where we found a significant increase in the number of somatostatin-expressing cells (Fig. 3o–p) and a dramatic reduction in the number of CCK-, glucagon-, and GLP-1-expressing cells (Fig. 3q–v). The number of



**Fig. 1.** Arx is expressed in the hormone producing cells scattered in the epithelium of the stomach and intestine. (a–f) Immunostaining analyses on E14.5 or E17.5 wild type stomach and intestine localize cells expressing Arx in the duodenal epithelium at E14.5 (a) and stomach (b), duodenum (c), jejunum (d), ileum (e) and colon (f) at E17.5. (g–h) Double immunostaining analysis on P3 wild type stomach (g) and duodenum (h) shows Arx is expressed in the chromograninA<sup>+</sup> enteroendocrine cells. (i–s) Double immunostaining analysis on wild type stomach and duodenum localize Arx expression to gastrin (i; 12/25 cells), glucagon (j; 7/17 cells), 5-HT serotonin (k; 1/2 cells), somatostatin (l; 2/13 cells), ghrelin (m; 8/18 cells) producing cells in the stomach and CCK (n), ghrelin (o), glucagon (p), GLP-1 (q), 5-HT serotonin (r), but not somatostatin (s) producing cells in the duodenum. “\*” denotes non-specific staining. Boxed areas in (i–m) show higher resolution of the images. Arx expression is not found in Paneth cells (t), tuft cells (u) and goblet cells (v–w). Scale bar: 50  $\mu$ m. Note: duodenum at P7 was used for glucagon/Arx co staining due to the low number of glucagon cells present in the duodenum of younger animals.



**Fig. 2.** *Arx*<sup>Endoderm</sup> mutant mice fail to thrive with abnormal ileal epithelium. (a–d) Immunostaining analysis on E17.5 control and *Arx*<sup>Endoderm</sup> mutant stomach and duodenum show efficient *Arx* removal. Arrows mark *Arx* expressing cells in control tissues (a, c). Scale bar: 50  $\mu$ m. (e) Daily body weight measurements of control and *Arx*<sup>Endoderm</sup> mutant mice from postnatal (P) day 0 to day 5. (f) P7 *Arx*<sup>Endoderm</sup> mutant mice appear smaller compared to their control littermates. (g) Survival graph shows approximately 50% of the *Arx*<sup>Endoderm</sup> mutant mice die during the first week of life. n = 43 (control) and n = 20 (*Arx*<sup>Endoderm</sup> mutant) mice were used at the beginning of the experiment. (h) *Arx*<sup>Endoderm</sup> mutants show dilated ileum (arrows). (i) Measurement of random fed glucose levels show P3 *Arx*<sup>Endoderm</sup> mutants suffer from relative hypoglycemia. (j–q) Hematoxylin/eosin staining of the P3 control and *Arx*<sup>Endoderm</sup> mutants reveal normal histology in the stomach (j–k), duodenum (l–m), and colon (p–q), but appearance of vacuoles in the ileum (n–o).



**Fig. 3.** Changes in the numbers of serotonin, somatostatin, gastrin, glucagon, GLP-1 and CCK-expressing cells in the *Arx*<sup>Endoderm</sup> mutant mice. (a–l) Immunostaining for chromogranin A (a–b), 5-HT serotonin (c–d), somatostatin (e–f), gastrin (g–h), glucagon (i–j) and ghrelin (k–l) in P3 control and *Arx*<sup>Endoderm</sup> mutant stomach with cell count averages from several animals. Cell numbers from corpus and antrum were counted, combined, and normalized to the total surface area. (m–z) Immunostaining for chromogranin A (m–n), somatostatin (o–p), CCK (q–r), glucagon (s–t), GLP-1 (u–v), 5-HT serotonin (w–x) and ghrelin (y–z) in P3 control and *Arx*<sup>Endoderm</sup> mutant duodenum with cell count averages from different mice. Cell numbers from duodenum were quantified and normalized to the total surface area. Bars represent means  $\pm$  SEM ( $n = 4$  for both groups. \* $p < 0.05$ , \*\* $p < 0.001$ ). Scale bar: 50  $\mu$ m. Note: Sections used for these experiments were not immediate adjacent sections; therefore, ChrA<sup>+</sup> cell number will not equal to the summation of all individual hormones examined. Also, ChrA does not label all enteroendocrine cells (Mellitzer et al., 2010).

serotonin- and ghrelin-expressing cells was unchanged as was the total number of ChrA-positive enteroendocrine cells (Fig. 3m–n and w–z). In addition, similar changes in the enteroendocrine cell types were observed in the P3 jejunum and ileum of *Arx*<sup>Endoderm</sup> mutant mice (Supple. Fig. 2 and data not shown). Real-time PCR analysis confirmed changes in expression levels of these hormone genes and

additionally observed a decrease in *TPH-1* and *secretin* mRNA levels in the P3 *Arx*<sup>Endoderm</sup> stomach and duodenum (Supple. Fig. 3a–b). While deficiency of several gastrointestinal transcriptional regulators including Pax4, Pax6, Nkx6.1, Nkx2.2, Nkx6.3, and Pdx1 has been shown to play a role in the development of enteroendocrine populations that were affected in the *Arx*<sup>Endoderm</sup> mice, we did not

detect any changes in the expression levels of any of these genes in the P3  $Arx^{Endoderm}$  stomach and duodenum (Supple. Fig. 3c–d).

#### *Arx is required in endocrine progenitors to specify cell fate choices*

$Arx$  is localized to a very few somatostatin cells in the stomach and is completely absent in these cells of the duodenum (Fig. 11 and s) and yet there is a dramatic increase in the number of this cell type in the  $Arx^{Endoderm}$  mutant mice (Fig. 3e–f and o–p). There are several possible explanations for the increase of somatostatin cell population seen in the  $Arx^{Endoderm}$  mutant mice, however one explanation is that  $Arx$  may play a cell fate decision role in the endocrine progenitors and that these cells become redirected into the somatostatin lineage when the  $Arx$ -dependent pathway is deleted. Alternatively, the increase in the somatostatin cell number could be due to fate switching from another enteroendocrine cell type upon  $Arx$  removal. To test the former possibility, we deleted  $Arx$  specifically in the  $Ngn3^+$  endocrine progenitors ( $Arx^{Endocrine}$  mutant mice) by intercrossing  $Ngn3-Cre$  mice to  $Arx$ -floxed mice (Fulp et al., 2008; Mastracci et al., 2011; Schonhoff et al., 2004). As enteroendocrine populations are known to show stereotypical variation across different regions of the stomach, (Fig. 4u; left image; (Jensen et al., 2000; Rindi et al., 2004)), we analyzed changes in enteroendocrine cell numbers specifically in the gastric corpus and antrum of P5 control and  $Arx^{Endocrine}$  mutant mice. Similar to what we have observed in the  $Arx^{Endoderm}$  mice, the number of somatostatin-expressing cells increased dramatically in the antrum, but not in the corpus (Fig. 4a–b). In contrast, glucagon- and gastrin-expressing cells were profoundly reduced in the corpus and antrum, respectively (Fig. 4c–f). We observed no significant increase in the numbers of serotonin-positive cells in the antrum or corpus; in contrast to what we had seen in the  $Arx^{Endoderm}$  mutant stomach (Figs. 3c–d and 4g–h). Lastly, we did not detect changes in the number of ChrA-expressing cells in either the antrum or corpus of  $Arx^{Endocrine}$  mutant mice (Fig. 4i–j). These data suggest that in the stomach  $Arx$  is required in the  $Ngn3^+$  endocrine progenitors to control cell fate choices for glucagon, gastrin and somatostatin, but not serotonin lineages. Alternatively, there may be an existence of endocrine precursor population that is positive for  $Arx$ , but devoid of  $Ngn3$  expression that can also give rise to serotonin lineage in the stomach (see below for further discussion).

In the duodenum of  $Arx^{Endocrine}$  animals, there was an increase in the numbers of somatostatin-expressing cells and a reduced number of glucagon-, GLP-1, and CCK-expressing cells, while the number of serotonin-expressing cells remained unchanged (Fig. 4k–t). The striking similarities in the changes of intestinal enteroendocrine cell populations between  $Arx^{Endoderm}$  and  $Arx^{Endocrine}$  mice strongly suggest that  $Arx$  is largely acting in endocrine progenitors to control specification of distinct subtypes of enteroendocrine lineages (Fig. 4u).

#### *GI dysfunction and alterations in enteroendocrine cell populations are observed in $Arx^{Intestine}$ mutant mice*

$Arx$  also plays a critical role in islet alpha cell development in the pancreas (Collombat et al., 2003) and pancreatic dysfunction has been postulated to contribute to the diarrhea seen in patients with ARX mutations (Itoh et al., 2010). To determine whether the  $Arx^{Endoderm}$  mutant intestinal phenotypes are independent of the known role of  $Arx$  in pancreas development, we generated intestine-specific  $Arx$ -deficient mice ( $Villin-Cre; Arx^{L/L}$  or  $Villin-Cre; Arx^{L/Y}$ ; referred to as  $Arx^{Intestine}$  mutant hereafter) by intercrossing  $Villin-Cre$  transgenic mice to  $Arx$ -floxed mice (el Marjou et al., 2004; Fulp et al., 2008). We found that although  $Arx^{Intestine}$  mutant mice have normal survival curves, they too suffered from chronic diarrhea with mildly reduced glucose levels compared to control mice at P3 ( $95 \pm 6.5$  mg/dl for controls and  $58 \pm 8$  mg/dl for mutants). These findings demonstrate that

intestinal  $Arx$  loss is the predominant cause for the diarrhea and hypoglycemia in  $Arx^{Endoderm}$  mice and that loss of  $Arx$  in the pancreas of these mutant mice only contributes to the hypoglycemia phenotype.

Immunostaining of intestinal tissue from adult  $Arx^{Intestine}$  mutant mice was performed which showed an increase in somatostatin-expressing cells and decreases in CCK, glucagon/GLP-1 cell populations in the duodenum (Supple. Fig. 4). Furthermore, analysis of the  $Arx^{Endoderm}$  mice that survived to adulthood showed that they continued to have soft stool and exhibited similar changes in the enteroendocrine populations in the stomach and duodenum (data not shown). These results suggest that although older mice continue to have chronic diarrhea likely due to enteroendocrine cell dysfunction, survival is not affected beyond early stages of their lives.

#### *Lipid accumulation in the ileum of $Arx^{Endoderm}$ and $Arx^{Intestine}$ mutant mice*

The cause of chronic diarrhea was next evaluated in Oil-red-O stained fecal samples from P3  $Arx^{Endoderm}$  and  $Arx^{Intestine}$  mutant mice, which showed an increased in staining indicating potential defects in the intestinal fat absorption (Fig. 5a–b and Supple. Fig. 5a–b). To further investigate this defect, different regions of the intestine were stained with Oil-red-O in which excess lipid accumulation was evident only in the ileum of  $Arx^{Endoderm}$  mutant mice (Fig. 5c–f and data not shown). These findings demonstrate that the abnormal vacuoles seen in the ileum of  $Arx^{Endoderm}$  were in fact lipid droplets (Fig. 2n–o). Excess lipid accumulation was also observed in the ileum of P3  $Arx^{Intestine}$  and  $Arx^{Endocrine}$  mutants (Supple. Fig. 5c–f and data not shown). Taken together, these findings suggest that intestinal  $Arx$  deficiency impacts development of several enteroendocrine cell populations, whose functions include the regulation of lipid processing and/or metabolism in the neonatal intestine.

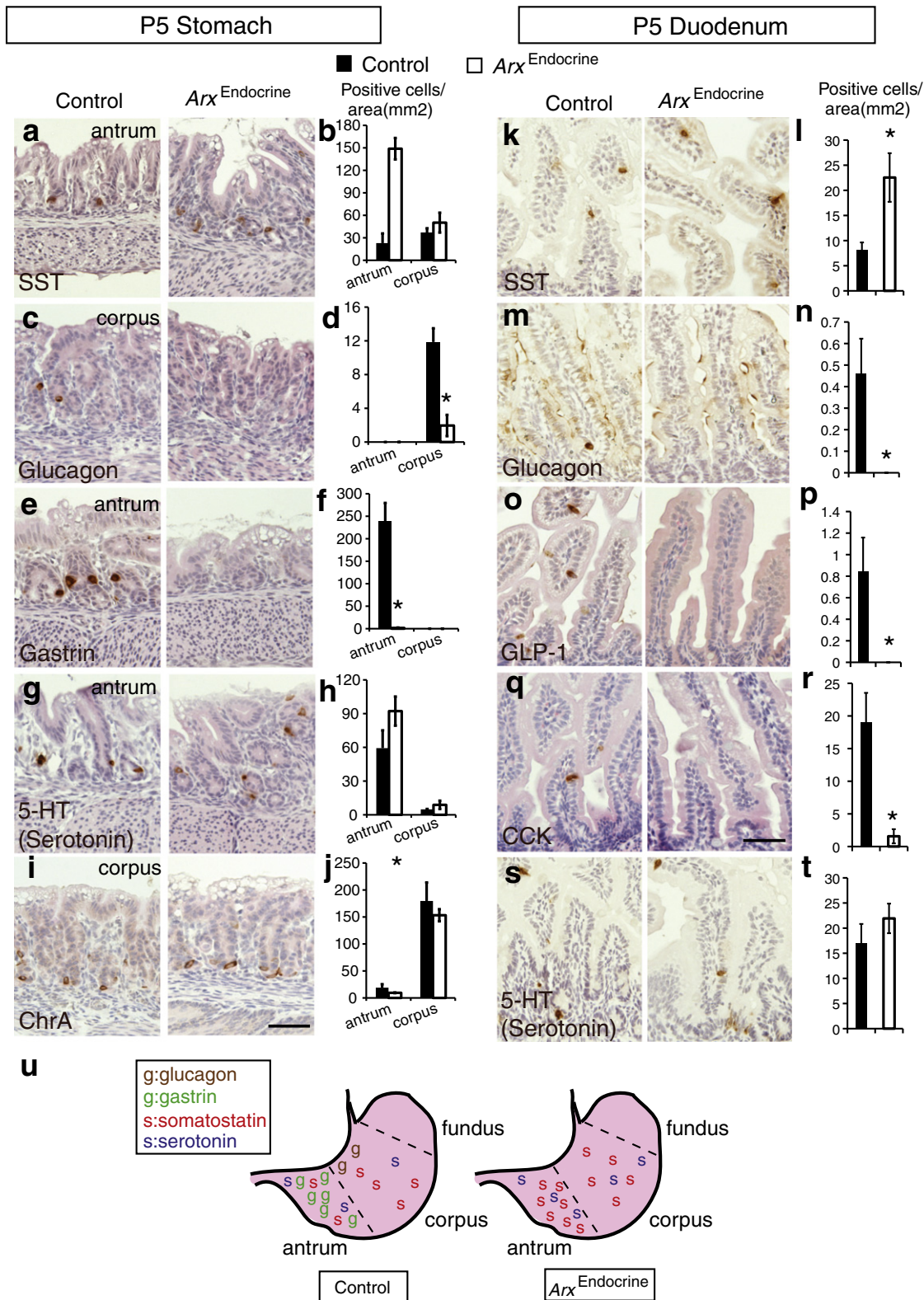
Lastly, since the goblet cell number was increased in the  $Ngn3$ -null mice at early postnatal stages and a possibility of goblet and enteroendocrine cells sharing part of their developmental program (Jenny et al., 2002; Jensen et al., 2000; Poulsom et al., 1993), we examined if there were changes in goblet cell population in our  $Arx^{Endoderm}$  mutant mice. Alcian blue stainings were performed and quantified in the intestine of P3 control and  $Arx^{Endoderm}$  mutant mice and showed no obvious changes in the numbers of goblet cells in the duodenum ( $2.25 \pm 0.16$  for control and  $2.39 \pm 0.32$  for mutant; per villus), ileum ( $4.59 \pm 0.21$  for control and  $4.27 \pm 0.34$  for mutant; per villus) or colon ( $10.22 \pm 0.37$  for control and  $10.54 \pm 1.2$  for mutant; per gland) (Fig. 5g–l). These observations indicate that  $Arx$  removal and/or altered enteroendocrine cell populations in the  $Arx^{Endoderm}$  mutant mice have no impact on the goblet cell development.

#### **ARX knock down in human intestinal tissue also causes enteroendocrine cell deficiency**

It has previously been reported that patients carrying ARX mutations suffer from diarrhea (Itoh et al., 2010; Kato and Dobyns, 2005), a phenotype that is also observed in  $Arx$ -deficient mice. To determine if this coincides with the loss of specific enteroendocrine cells as it does in mice, we investigated if ARX is required for normal human enteroendocrine cell development. We performed shRNA-mediated loss-of-function in human intestinal organoids (HIOs) derived from pluripotent stem cells via directed differentiation (Spence et al., 2011). To generate stable shRNA-ARX knockdown lines, human ES cells were transduced with lentivirus expressing ARX shRNA and differentiated into proximal intestinal organoids, which had between 50 and 60% reduction in ARX mRNA levels (Fig. 6a). HIOs were analyzed at day 68, at which time all intestinal cell types can be observed (Spence et al., 2011). Immunostaining analysis showed normal CDX2 expression in epithelium of the ARX knockdown organoids as compared to control organoids, which

indicates that ARX knockdown did not inhibit the formation of a CDX2-positive epithelium (Fig. 6b–c). Additionally, ARX knockdown did not affect expression levels of markers for enterocytes (*Lactase*),

goblet cells (*Muc2*), or Paneth cells (*Lysozyme*) (Fig. 6d–f). Furthermore, there were no changes in the endocrine markers *CHRA* or *Ghrelin*; however, ARX knockdown did cause a 60–80% reduction in



**Fig. 4.** Arx is required in the endocrine progenitors to control cell fate decisions. Immunostaining analysis on P5 control and *Arx<sup>Endocrine</sup>* mutant stomach and duodenum for somatostatin (a–b, k–l), glucagon (c–d, m–n), gastrin (e–f), GLP-1 (o–p), and CCK (q–r), 5-HT serotonin (g–h, s–t) and ChrA (i–j), for the stomach, cell numbers were quantified and normalized to the epithelial area separately for antrum and corpus. For the duodenum, total cell numbers were counted and normalized to the total epithelial area. Bars represent means ± SEM. (n = 4, \*p < 0.05, \*\*p < 0.001). Scale bar: 50 μm. (u) The drawing summarizes the observed regional changes of enteroendocrine populations in the stomach. Note: Sections used for these experiments were not immediate adjacent sections; therefore, ChrA<sup>+</sup> cell number will not equal the summation of all individual hormones examined. Also, ChrA does not label all enteroendocrine cells (Mellitzer et al., 2010).



CCK, Secretin, and Preproglucagon mRNA levels (Fig. 6g–l) demonstrating that development of a subset of enteroendocrine cells is dependent on the presence of ARX in humans as it is in mice. Interestingly, *Somatostatin* gene expression was not increased in ARX depleted human organoids as it was in *Arx*-deficient mice (Fig. 6k, Fig. 3o–p, 4k–l, Supple Figs. 3b, 4a–b). These results suggest that human enteroendocrine cell development requires ARX and the GI dysfunction in patients with ARX mutations may be caused by altered numbers of enteroendocrine cells in the GI tract.

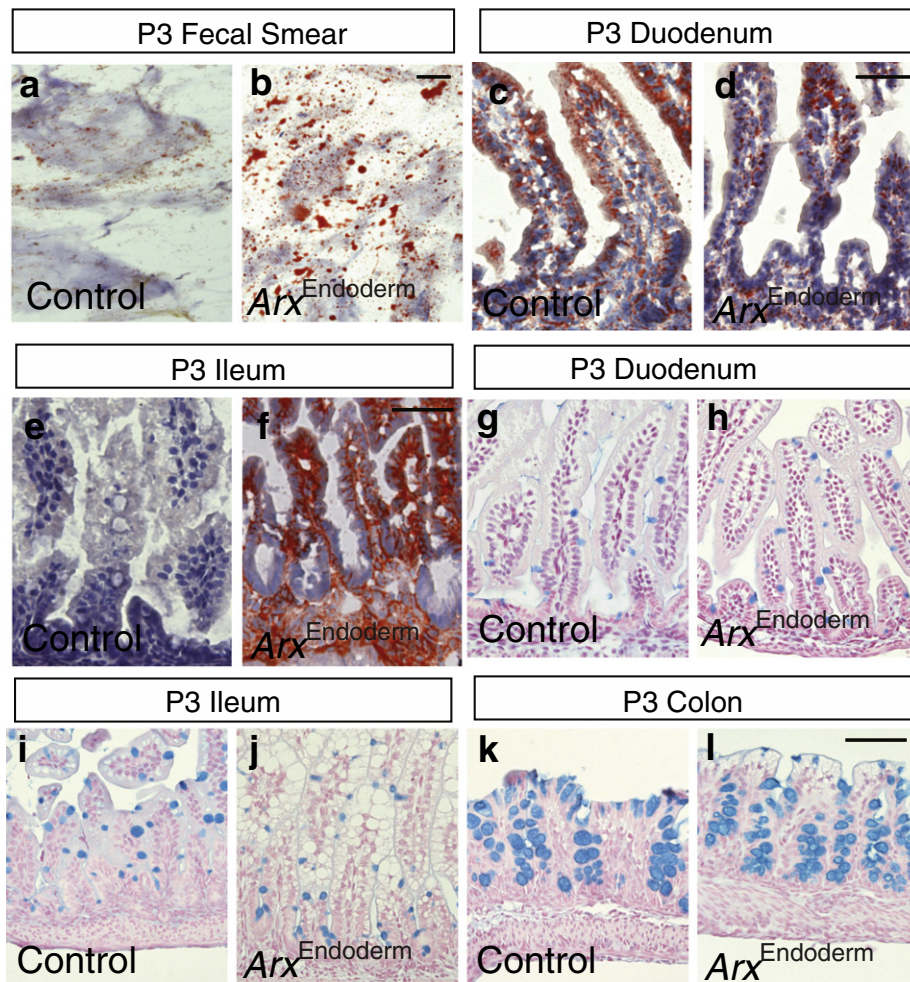
## Discussion

The function of *Arx* has been identified and studied extensively in the brain and endocrine pancreas (Collombat et al., 2003, 2005, 2007; Colombo et al., 2007; Friocourt et al., 2008; Hancock et al., 2010; Kitamura et al., 2002; Marsh et al., 2009; Mastracci et al., 2011), yet its role in developing GI endocrine cells has not been characterized. Findings from our study not only contribute vastly to the body of knowledge on transcriptional control of enteroendocrine cell development in mice and humans, they also provide insights into the pathogenesis of GI symptoms observed in patients with ARX mutations.

Our analyses of mice deficient for *Arx* in the developing endoderm (*Arx*<sup>Endoderm</sup>) and in the intestine (*Arx*<sup>Intestine</sup>) show changes in the

enteroendocrine populations, which are likely to result in hormone imbalance, leading to severe diarrhea. In addition, removing *Arx* in *Ngn3*<sup>+</sup> endocrine progenitors (*Arx*<sup>Endocrine</sup>) further demonstrates that *Arx* is required for cell fate decisions in these cells. In fact, similar changes in the numbers of gastric somatostatin, gastrin and glucagon cells and intestinal somatostatin, CCK, glucagon/GLP1 cells were observed in the *Arx*<sup>Endocrine</sup> and *Arx*<sup>Endoderm</sup> mutants. Although our findings do not directly demonstrate that *Arx* is involved in favoring certain enteroendocrine lineages at the expense of the others within the endocrine progenitor population as demonstrated in the *Arx*-deficient pancreas (Collombat et al., 2003, 2005), these observations are suggestive of such a possibility in the intestine based on the lack of *Arx* expression in the somatostatin cells and a profound increase of this cell type upon *Arx* removal in the endocrine progenitors.

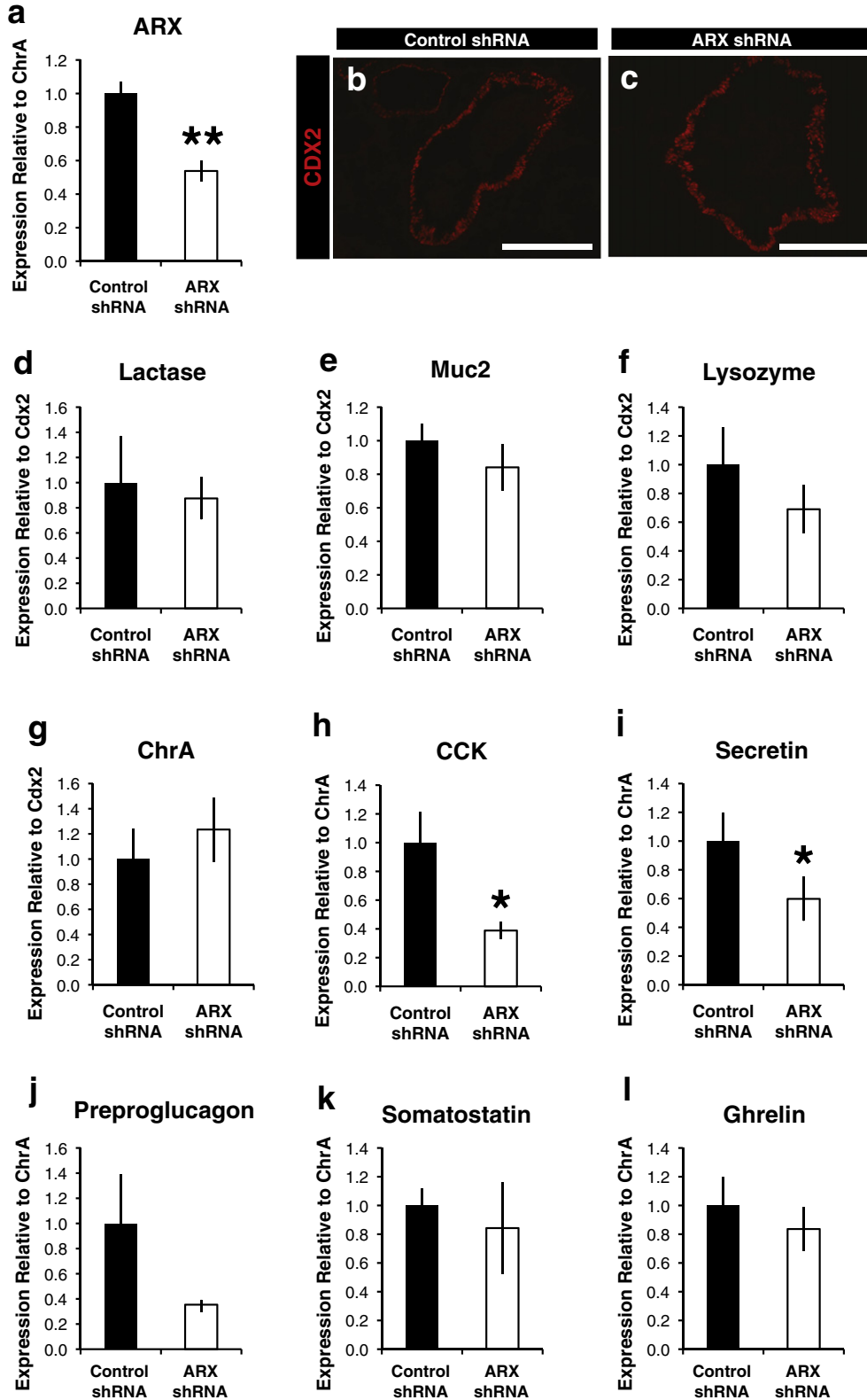
The importance of enteroendocrine progenitors, mature enteroendocrine cells, and hormones secreted by these cells have been suggested to play a critical role in maintaining overall homeostasis of the intestinal crypt compartment (Mellitzer et al., 2010). In contrast to the altered cell homeostasis observed in the *Ngn3*-deficient adult intestine (Mellitzer et al., 2010), we did not detect any disorganization in the crypts in the neonatal or adult *Arx*-deficient mice suggesting that the affected enteroendocrine populations in the *Arx*-deficient mice may not play a role in maintaining the crypt homeostasis. Furthermore, in contrast to an increase in the goblet



**Fig. 5.** Gastrointestinal epithelium appears normal with fat accumulation in the ileum of P3 *Arx*<sup>Endoderm</sup> mutants. (a–b) Oil-red-O staining of fecal smear. (c–f) Oil-red-O staining of the P3 duodenum and ileum demonstrates presence of increased fat accumulation only in the ileum of *Arx*<sup>Endoderm</sup> mutants. (g–l) Alcian blue staining of the duodenum, ileum and colon reveals no significant changes in the numbers of goblet cells between *Arx*<sup>Endoderm</sup> mutant and control mice. Scale bar: 50  $\mu$ m.

cell number observed in the *Ngn3* null mice (Jenny et al., 2002), we also did not detect changes in this cell type in the intestine of the *Arx*<sup>Endoderm</sup> mice suggesting that *Arx* is not involved in regulating

part of the developmental program that might be shared by enteroendocrine and goblet cells as previously suggested (Jenny et al., 2002; Jensen et al., 2000; Poulsom et al., 1993).



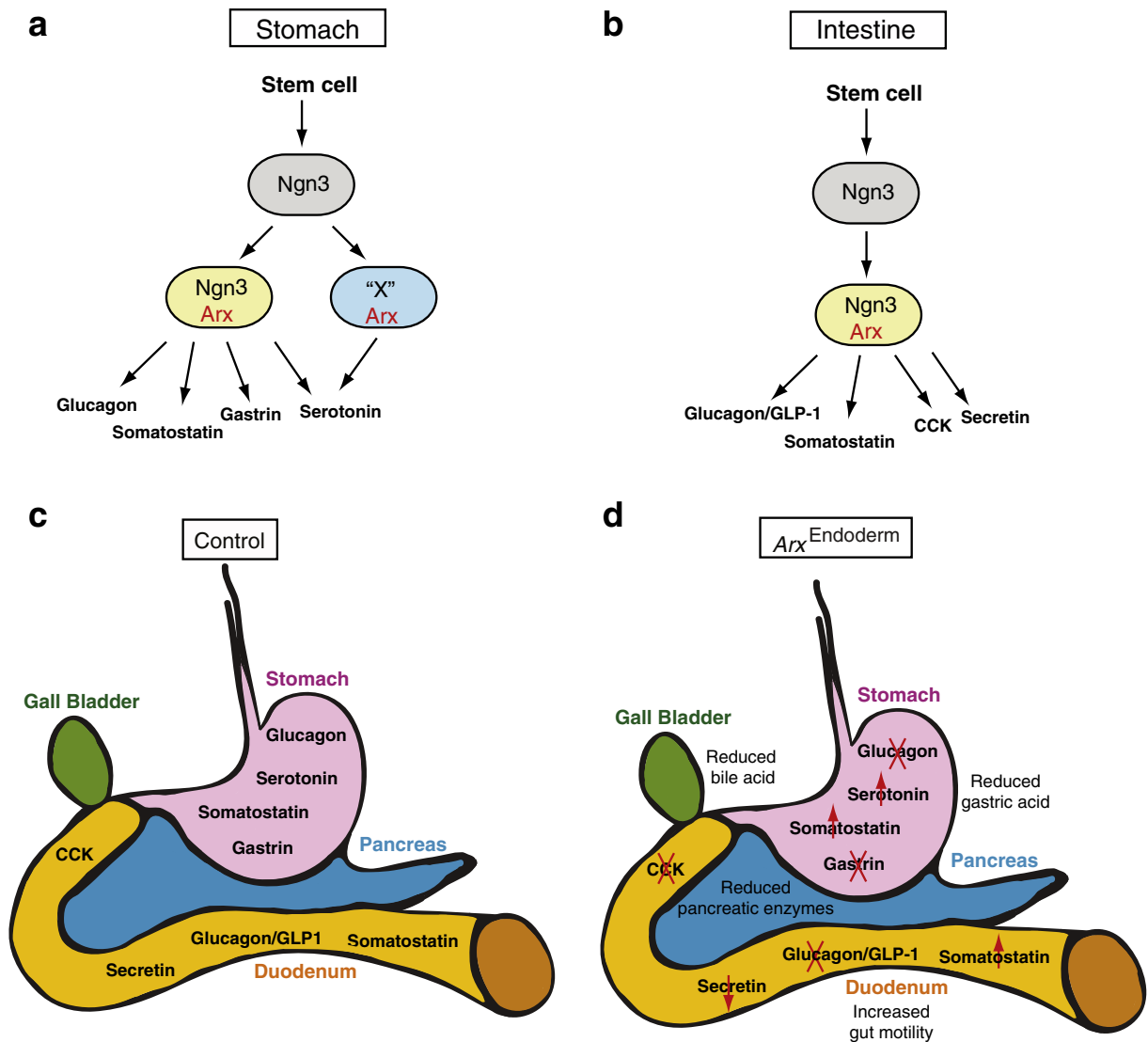
**Fig. 6.** Human enteroendocrine cell development requires ARX. Proximal intestinal tissue was generated from human embryonic stem cell lines and analyzed at 68 days after differentiation. Stable lines containing ARX shRNA had between 50 and 60% reduction in ARX mRNA (a) and a similar reduction in the proximal enteroendocrine cell markers CCK (h), secretin (i), and preproglucagon (j). There was no significant change in the pan-endocrine marker *ChrA* (g) or the enteroendocrine cell markers *somatostatin* and *ghrelin* (k, l). Overall, ARX knockdown did not affect intestinal development, as there were no differences observed in the pan-epithelial marker CDX2 (b, c) or differentiation of enterocytes (*lactase*, d), goblet cells (*MUC2*, e), or Paneth cells (*lysozyme*, f). Data were analyzed using a Student's *t*-test. \**p*<0.05. \*\**p*<0.01. Scale bars in b, c represent 200  $\mu$ m.

While in *Arx*-deficient mice the gross architecture of the crypts and villi in the gastrointestinal epithelium appears normal with presence of all intestinal cell types, an abundance of lipid-filled enterocytes was detected in the ileum of *Arx*<sup>Intestine</sup>, *Arx*<sup>Endoderm</sup> and *Arx*<sup>Endocrine</sup> mutant mice at early postnatal stages. This phenotype is reminiscent of the intestinal phenotype found in the *Pleomorphic adenoma gene-like 2 (Plagl2)* deficient pups in which lipid-filled enterocytes were found in the jejunum (Van Dyck et al., 2007). Since *Plagl2* is expressed in the enterocytes and *Arx* is only found in the enteroendocrine cells, our findings suggest that enteroendocrine subpopulations affected in the *Arx*-deficient mice are likely hormone cell types that are involved in regulating lipid absorption and/or transport. Since no obvious morphological alterations or lipid-filled enterocytes were found in the proximal intestine of the *Arx*-deficient mice, the lipid transport system is likely overloaded and only affected profoundly in the distal region of the intestine similar to what was described in the distal jejunum of *Plagl2* mutants at nursing ages (Van Dyck et al., 2007). Although we cannot identify the specific hormones that are responsible for regulating gut lipid metabolism, our

findings suggest that combined changes in somatostatin, CCK, secretin and glucagon/GLP1 populations are likely impacting this process.

Interestingly, although similar hormone populations were affected in the adult *Arx*-deficient mice, we did not detect the presence of vacuoles in the ileal enterocytes in mice that were fed normal chow (data not shown) suggesting that there is an age- and diet-specific impact on hormone deficiency and lipid metabolism in the *Arx* mutants. As milk contains higher fat content than the solid food that the adult mice consume, it would be interesting to see whether placing adult *Arx*-deficient mice on a high fat diet would also lead to an increase in the number of lipid-filled intestinal enterocytes similar to what was observed in the nursing pups.

In contrast to the *Arx*<sup>Endoderm</sup> mutants which showed chronic diarrhea and reduced survival, *Arx*<sup>Endocrine</sup> mutant mice had a normal survival curve and did not suffer from diarrhea even though similar hormone populations, except gastric serotonin cells, were found to be affected profoundly in the stomach and intestine. It is important to keep in mind that there are at least 15 enteroendocrine subtypes present in the GI tract and *Arx* is selectively removed in the *Ngn3*<sup>+</sup>



**Fig. 7.** Models for the proposed role of *Arx* during enteroendocrine cell differentiation and the summary of the observed cell population changes and processes affected in *Arx*<sup>Endoderm</sup> mutant mice. (a) *Arx* is expressed in the *Ngn3*<sup>+</sup> progenitors (yellow) for the development of gastric glucagon, somatostatin and gastrin cells. However, serotonin cells can arise from either *Arx*/*Ngn3* lineage (yellow) or another precursor expressing *Arx* and an unidentified factor X (blue). All these enteroendocrine subtypes are probably derive from *Ngn3*<sup>+</sup> endocrine progenitors (gray). (b) *Arx* is expressed in the *Ngn3*<sup>+</sup> endocrine progenitors (yellow) for the development of somatostatin, glucagon/GLP-1, CCK and secretin cells in the intestine. (c–d) *Arx*<sup>Endoderm</sup> mutant mice display increased gut motility resulting from a reduced number of gastrin-, CCK-, glucagon/GLP-1-, and secretin-expressing cells and an increase in somatostatin cells (d) relative to control littermates (c). Changes in these cell types result in a reduction of gastric acid, pancreatic enzymes, and bile acids.

endocrine progenitors in the  $Arx^{Endocrine}$  mutants and in all epithelial cells in the  $Arx^{Endoderm}$  mutants. Furthermore, it has been suggested that gastric serotonin cell development is controlled in *Ngn3*-dependent and independent fashions as this cell type is not completely missing in the stomach of *Ngn3* null mice (Jenny et al., 2002; Lee et al., 2002). Consistent with this model and if *Ngn3*-Cre activity in the  $Arx^{Endocrine}$  mutants was delayed and only present in *Ngn3*/*Arx* endocrine progenitors of the stomach (yellow cell; Fig. 7a), then the serotonin cell population would only be partially affected in the  $Arx^{Endocrine}$  mutants and more profoundly impacted in the  $Arx^{Endoderm}$  mutants. In fact, this is exactly what we observed in our immunostaining analyses for serotonin cells in the stomach of these two mutants. Additionally, some unexamined enteroendocrine cells in the stomach may also be selectively affected only in the  $Arx^{Endoderm}$  mutants. Therefore, a global gene expression analysis (e.g. RNA-seq) will be most informative to thoroughly determine if additional genes associated with enteroendocrine subpopulations were uniquely affected in the  $Arx^{Endoderm}$  mutants.

The chronic diarrhea seen in the  $Arx^{Intestine}$  and  $Arx^{Endoderm}$  mice is also apparent in subset of patients with *ARX* mutations who not only suffer from a wide variety of neurologic conditions, including X-linked lissencephaly with abnormal genitalia (XLAG), but also have a reduced number of pancreatic endocrine and exocrine cells (Bienvenu et al., 2002; Gecz et al., 2006; Itoh et al., 2010; Kitamura et al., 2002; Stromme et al., 2002a,2002b; Kato and Dobyns, 2005). The shortened life span of these patients and mice with whole body *Arx* mutations has been attributed to profound neurologic dysfunction or the complete absence of glucagon-producing alpha-cells in the pancreas instead of endocrine cell dysfunction in the intestine (Collombat et al., 2003; Gecz et al., 2006; Hancock et al., 2010; Marsh et al., 2009). Our data suggest that gastrointestinal defects in patients with *ARX* mutations may also contribute to the overall degenerative condition in these patients.

It has been proposed that a deficiency in both pancreatic polypeptide and pancreatic exocrine enzymes contributes to the intractable diarrhea in *ARX* patients (Itoh et al., 2010). However, we did not detect a change in the number of pancreatic polypeptide-producing PP cells nor was there a reduced amount of pancreatic amylase staining in our  $Arx^{Endoderm}$  mutant mice (data not shown). In addition, our analysis of the  $Arx^{Intestine}$  mutants, which showed similar GI dysfunction further provides evidence that the diarrhea observed in the mice as well as the humans more likely stems from changes in the GI hormone levels that result in loss of regulated release of digestive enzymes from the exocrine pancreas.

Although animal models are ideal for identifying functions of genes of interest, the use of hES cell-derived human intestinal tissue has allowed us to identify both conserved and human-specific functions of *ARX* during intestinal endocrine cell differentiation. For example, *ARX* is required in both mice and humans for normal development of CCK, secretin and glucagon/GLP-1-expressing cells. In contrast, *ARX* is not required for development of human somatostatin-expressing cells in the proximal intestine, similar to what has been shown in the human pancreas (Itoh et al., 2010). These findings not only provide valuable information on basic enteroendocrine cell development but also emphasize the importance of cell-based model systems to study human diseases as human tissues are often difficult to obtain for such studies.

*Arx* is acting as a key factor in the endocrine progenitors during enteroendocrine cell differentiation. Even though there are many more endocrine subtypes present in the GI epithelium as compared to the endocrine pancreas, *Arx* is also involved in promoting the glucagon/GLP-1 cell populations and repressing the somatostatin cell population in the intestine (Fig. 7b–d). Additionally *Arx* is also required for the development of CCK and secretin cells, two endocrine subtypes that are unique to the intestine (Fig. 7b–d). Interestingly, in the stomach, an increase in the somatostatin cell population was

only detected in the antrum of  $Arx^{Endocrine}$  mutant mice, a region that is devoid of glucagon cells and filled with gastrin cells. The decrease in the gastrin cell number and the increase in the somatostatin cell number were suggestive of a possibility that *Arx* might be involved in promoting gastrin and repressing somatostatin cell differentiation simultaneously in the antral stomach. The potential of these two enteroendocrine populations sharing a common precursor in the stomach is further supported by the presence of gastrin and somatostatin co-expressing cells in addition to the cells expressing either gastrin or somatostatin alone (Larsson et al., 1995). The development of the gastrin and somatostatin cells has also been suggested to be regulated by *Isl-1* (*Isl-1*), a LIM-homeodomain transcription factor (Larsson et al., 1995). In addition, *Isl-1* plays a key role in regulating *Arx* expression in the islet alpha cells as *Isl-1* removal in the embryonic pancreas results in a decrease of *Arx* expression (Du et al., 2009; Liu et al., 2011). Based on these observations, we hypothesize that differentiation of gastrin and somatostatin cells is likely controlled by a combinatorial role of *Isl-1* and *Arx* as expression of these two transcription factors overlaps in the stomach (unpublished data). Lastly, lineage tracing studies will be required in the future to determine the relationship between all the hormone populations that were affected in the *Arx*-deficient mice and if *Arx* promotes one enteroendocrine population at the expense of the other.

It has been shown that not all cells express equal levels of transcription factor as demonstrated in the pancreas for *Pdx1* and *Ngn3* (Fujitani et al., 2006; Nishimura et al., 2006; Villasenor et al., 2008; Wang et al., 2009). Our expression analyses show that *Arx* is only found in subsets of *Ngn3*<sup>+</sup> endocrine progenitors and hormone-producing cells. One possible explanation is the potential presence of  $Arx^{HI}$  and  $Arx^{LO}$  cells that are at different stages or ages within the enteroendocrine population. In order to determine whether  $Arx^{HI}$  and  $Arx^{LO}$  cells truly exist in the GI epithelium, additional *Arx* antibodies with higher sensitivities or assessment of *Arx* expression via LacZ staining in the  $Arx^{+/LacZ}$  mice (Collombat et al., 2003) will be required. Overall, our findings reveal a novel role for *Arx* in the endocrine progenitor during enteroendocrine development. Future studies on *Arx* in the GI tract will help determine whether *Arx* is also required at later stages to maintain the mature phenotypes of this population and to determine the molecular mechanisms by which *Arx* is involved in regulating this process including regulation of hormone gene expression.

## Conclusion

Here we demonstrate that *Arx* is required in the endocrine progenitors to specify particular enteroendocrine cell fates. The enteroendocrine populations affected in the absence of *Arx* are critical in regulating digestion (gastrin, somatostatin, CCK and secretin) and gut motility (serotonin and GLP-1). Therefore, changes in the number of these cell types may affect overall physiology, including release of gastric acid, bile, and digestive enzymes, and rate of chyme transit in these mice (Fig. 7c–d) (Baggio and Drucker, 2007; Beglinger and Degen, 2004; Drucker, 2007a; 2007b; Gershon, 1968; Lloyd, 1994; Mellitzer and Gradwohl, 2011; Murphy and Bloom, 2006; Murphy et al., 2006; Rehfeld, 2004a; 2004b; Schubert, 2010; Wren, 2008; Wren and Bloom, 2007; Wren et al., 2001).

Our study also provides evidence that the lack of specific enteroendocrine cell types in the GI tract is likely the primary cause of GI dysfunction seen in patients with *ARX* mutations. These findings will provide a thorough understanding for designing more efficient differentiation protocols toward generation of specific enteroendocrine types in vitro and generating effective treatments for patients with *ARX* mutations.

## Competing financial interests

The authors declare no competing financial interests.

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