The Homeodomain-Containing Transcription Factors Arx and Pax4 Control Enteroendocrine Subtype Specification in Mice

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Abstract

Intestinal hormones are key regulators of digestion and energy homeostasis secreted by rare enteroendocrine cells. These cells produce over ten different hormones including GLP-1 and GIP peptides known to promote insulin secretion. To date, the molecular mechanisms controlling the specification of the various enteroendocrine subtypes from multipotent Neurog3+ endocrine progenitor cells, as well as their number, remain largely unknown. In contrast, in the embryonic pancreas, the opposite activities of Arx and Pax4 homeodomain transcription factors promote islet progenitor cells towards the different endocrine cell fates. In this study, we thus investigated the role of Arx and Pax4 in enteroendocrine subtype specification. The small intestine and colon of Arx- and Pax4-deficient mice were analyzed using histological, molecular, and lineage tracing approaches. We show that Arx is expressed in endocrine progenitors (Neurog3+) and in early differentiating (ChromograninA+) GLP-1+, GIP+, CCK-, Sct- Gastrin- and Ghrelin-producing cells. We noted a dramatic reduction or a complete loss of all these enteroendocrine cell types in Arx mutants. Serotonin- and Somatostatin-secreting cells do not express Arx and, accordingly, the differentiation of Serotonin cells was not affected in Arx mutants. However, the number of Somatostatin-expressing D-cells is increased as Arx-deficient progenitor cells are redirected to the D-cell lineage. In Pax4-deficient mice, the differentiation of Serotonin and Somatostatin cells is impaired, as well as of GIP and Gastrin cells. In contrast, the number of GLP-1 producing L-cells is increased concomitantly with an upregulation of Arx. Thus, while Arx and Pax4 are necessary for the development of L- and D-cells respectively, they conversely restrict D- and L-cells fates suggesting antagonistic functions in D/L cell allocation. In conclusion, these findings demonstrate that, downstream of Neurog3, the specification of a subset of enteroendocrine subtypes relies on both Arx and Pax4, while others depend only on Arx or Pax4.

Introduction

Enteroendocrine cells belong to one of the four main intestinal cell subtypes, including enterocytes, goblet and Paneth cells, and represent about 1% of all epithelial cells. These cells secrete various amine and peptide hormones and are classified according to their main secretory product, including Glugagon-like peptide 1 (GLP-1), Glugagon-like peptide 2 (GLP-2) and Peptide YY (PYY) secreted by L-cells, Gastric inhibitory peptide (K-cells), Somatostatin (D-cells), Cholecystokinin (I-cells), Secretin (S-cells), Gastrin (G-cells), Serotonin (EC cells), and Neurotensin (N-cells). The gastric peptide Ghrelin is also found in the small intestine and colon [1,2], but it remains unclear whether intestinal Ghrelin-expressing cells constitute a separate enteroendocrine subtype. Intestinal hormones control numerous physiological functions, such as glucose homeostasis for the Glucoincretin GLP-1 and GIP, food intake, pancreatic and gastric secretion, or gastrointestinal mobility [3,4]. In mice, the loss of all enteroendocrine cells leads to growth retardation, impaired lipid absorption and increased lethality, underlying the importance of enteroendocrine function [5].

During the course of development, enteroendocrine cells, as well as the two other secretory cell types, goblet and Paneth cells, arise from intestinal stem cells through an intermediate progenitor expressing the basic helix-loop-helix (bHLH) transcription factor (TF) Atoh1 [6,7]. The specification of this Atoh1+ secretory progenitor cell towards the endocrine lineage is controlled by the bHLH TF Neurog3 that also determines endocrine cell destiny in the stomach and pancreas [8,9,10]. Downstream of Neurog3, several TFs have been shown to be required for proper enteroendocrine cell differentiation. Among these, the zinc-finger TF Insml is necessary for generic features of endocrine cells as well as for the differentiation of particular subtypes. Indeed, targeted disruption of Insml leads to the loss of expression of Chromogranin A (ChgA) secretory vesicle protein [11]. Hormone
expression is only partially affected by the absence of Insm1. Substance P and Neurotensin (Ns) cells are lost but the numbers of Serotonin (5HT), Cholecystokinin (CCK) and PYY cells are only reduced. Additional TFs were found to control the allocation towards specific enteroeendocrine subtypes. Thus, NeuroD1 controls the differentiation of Secretin (ScI) and CCK cells [12], whereas Foxa1 and Foxa2 promote the differentiation of GLP1- and Somatostatin (Sst)-expressing cells [13]. The NK-homeodomain-encoding gene, Nkx2.2, is necessary for CCK, Gastrin, Gastric Inhibitory Polypeptide (GIP), Nts and Sst expression [14]. The paired-box transcription factors Pax4 and Pax6 also control enteroeendocrine cell differentiation. Although Pax6 knockout phenotype has not been extensively investigated in the intestine, it has been reported that GIP cells are Pax6-dependant [15]. Other studies indicate that Pax6 acts downstream and in concert with Foxa1 and Foxa2 to regulate the transcription of the preproglutaglutamidase [16]. Importantly, both Pax4 and Pax6 are required for enteroeendocrine cell differentiation. NeuroD1 is expressed in Neurog3-deficient embryonic intestines (data not shown) and Neurog3 is necessary for the differentiation of GLP-1- and Nts-expressing cells in the adult intestine [17]. NeuroD1-deficient mice for Pax4 lack beta- and delta-cells and display a concomitant increase in alpha-cell number [16]. Conversely, Arx inactivation leads to an opposite phenotype, characterized by an absence of alpha-cells and an increase in the number of beta- and delta-cells [17]. Furthermore, the forced expression of Arx in enteroeendocrine progenitors induces their specification towards the alpha-/PP-cell lineages at the expense of the beta-/delta-cell fates [18]. Interestingly, the ectopic expression of Pax4 in alpha-cells is sufficient to convert these cells into beta-like cells [19]. Therefore, the decision between the alpha-/PP- or beta-/delta-cell fate seems to be mainly directed by the cross-repression of Pax4 and Arx genes [20]. Thus, the balance between Arx and Pax4 in pancreatic endocrine progenitors plays a key role in endocrine subtype allocation.

Since Arx and Pax4 control islet subtype destiny in the developing pancreas, we postulated that similar mechanisms could govern cell fate choices in the enteroeendocrine lineage. In this study, we therefore investigated the function of Arx and Pax4 in the intestine. Our results indicate that Arx is restricted to the enteroeendocrine lineage and downstream of Neurog3. Importantly, Arx is required for the differentiation of a subset of enteroeendocrine cells. Indeed, Arx-deficient mice display an almost complete loss of GLP1, GIP, CCK and Nts cells, with a concomitant increase in Sst-expressing cell numbers. On the other hand, Pax4-deficient mice lack Nts, Glp1 and Serotonin cells, whereas GLP1 cell number is significantly increased. Taken together, these results indicate that while Arx and Pax4 are similarly required for the proper differentiation of a subset of enteroeendocrine cells, they differentially regulate the development of specific enteroeendocrine cells. In contrast to the embryonic pancreas antagonistic functions of Arx and Pax4 seems limited to the control of L (GLP-1)- and D (Sst)-cell differentiation.

Results

Arx is transiently expressed in a subset of developing enteroeendocrine cells

To characterize the expression pattern of Arx in the embryonic and adult mouse intestine, we combined quantitative RT-PCR, in situ hybridization and double immunohistochemistry using antibodies raised against Arx, Neurog3, ChromograninA, and intestinal peptides. In the adult wild-type intestine, Arx transcripts are revealed from the duodenum to the colon (Fig. 1A). Importantly, Arx transcripts cannot be detected in the duodenum of Villin-Cre; Neurog3<sup>fl/fl</sup> mice (Fig. 1B), which lack enteroeendocrine cells [3]. This suggests that, like in the pancreas [17], Arx expression remains restricted to the enteroeendocrine lineage in the intestine. Accordingly, scattered Arx<sup>+</sup> cells are found throughout the adult intestine in a pattern reminiscent of enteroeendocrine cells (Fig. 1C, S1). In the small intestine, Arx is expressed in post-mitotic crypt cells (Fig. S2), mainly in subsets of Neurog3<sup>+</sup> cells (Fig. 1D), suggesting that Arx expression is initiated in enteroeendocrine progenitor cells. Arx is not detected in mature Chga<sup>+</sup> endocrine cells (Fig. 1C), however cells double-positive for Arx and intestinal peptides GLP1, GIP, CCK, Gastrin or Glhrelin (Ghrl) are present within the crypts, supporting the notion that Arx expression is maintained in early differentiating L-, K-, I-, G- and Ghrelin-cells (Fig. 2). As Arx-positive cells migrate during their differentiation to reach the base of the villus, Arx expression progressively diminishes and eventually vanishes (Fig. 2 compare A to B), further suggesting that Arx is expressed in nascent but not mature hormone-expressing cells. Importantly, Arx is never detected in Somatostatin- or Serotonin-expressing D or EC cells respectively (Fig. 2). During embryogenesis, at E14.5 when enteroeendocrine commitment is initiated in Neurog3<sup>+</sup> cells, Arx expression is not detectable. However, around E15.5, Arx-expressing cells emerge in the embryonic intestine, at a stage corresponding to the onset of enteroeendocrine differentiation (Fig. 1E). Arx transcripts are not detected in Neurog3-deficient embryonic intestines (data not shown) and thus, like in the adult, Arx expression is restricted to the enteroeendocrine lineage. Taken together, these data indicate that in the embryonic intestine Arx lies downstream of Neurog3 in enteroeendocrine committed cells. In the adult intestine Arx appears transiently expressed downstream of Neurog3 in endocrine progenitors and developing, but not fully differentiated, L-, K-, I-, G- and Ghrelin-cells, whereas D- and EC-cells do not appear to arise from Arx<sup>+</sup> precursors.

Enteroeendocrine cell differentiation is severely impaired in Arx-deficient mice

We next analyzed enteroeendocrine cell differentiation in Arx-deficient mice. Arx mutants do not survive beyond postnatal day 2 (P2). We therefore examined intestinal hormone expression combining real-time PCR (Fig. 3A and Table S1) with immunofluorescence (Fig. 3B, S3) at P1-P2. Arx-deficient mice display an almost complete absence of Glp1<sup>+</sup>, Gip<sup>+</sup>, Cck- and Nts-expressing cells in the small intestine and colon (Fig. 3 and S3A), whereas Set and Gastrin mRNA levels are significantly diminished. Pyy<sup>+</sup> mRNA expression, a marker of Serotonin-expressing EC cells, is unchanged. In agreement with the RT-QPCR data, the numbers of Serotonin-expressing cells are similar in controls and mutants (Fig. 3B). Interestingly, Chga expression is also unaffected in the Arx mutant intestine, suggesting that the overall number of enteroeendocrine cells is not altered. As suggested by the restriction of Arx expression to the enteroeendocrine lineage, Arx inactivation does not alter the differentiation of Goblet cells (Fig. 4 and data not shown). Thus, our results demonstrate that Arx is necessary for the differentiation of GLP1<sup>+</sup>, Gip<sup>+</sup>, Cck<sup>+</sup>, Sct<sup>+</sup>, Gastrin<sup>+</sup>- and Nts-expressing cell lineages and...
suggest that failed cells, would to some extend, develop into $Sst^+$ and $Ghrl^+$ cells.

**Arx-deficient progenitors are reallocated to Somatostatin-expressing cells**

To determine whether the changes in endocrine differentiation observed upon Arx deficiency were caused by alternative fate specification, we analyzed the expression of intestinal hormones in Arx-deficient cells. In mice carrying an Arx mutant allele, the beta-galactosidase (beta-gal) protein is expressed under the control of Arx regulatory elements. Due to the location of Arx on the X chromosome, random X inactivation leads to the silencing of either the wild-type or $LacZ^-$ Arx allele in heterozygous females ($X^{wt}/*$ or $X^{LacZ}-/X^{LacZ}$). Consequently, in $X^{wt}/*$ females, cells expressing the Arx protein do not express the beta-gal, and cells expressing the beta-gal are Arx-deficient. In the adult intestine of $X^{wt}/*$ female mice, we did not detect any beta-gal expression in GLP1-, GIP-, or CCK-producing cells (Fig. 5A), confirming that Arx expression is required for the generation of $L$-, $K$-, and $I$-cells. In contrast, beta-gal was found in a subset of $Sst$-producing cells (Fig. 5A), which do not express Arx in wild-type intestine (Fig. 2). Together with the observed increase of $Sst$ mRNA (Fig. 3A) and the augmentation of $Sst$-expressing cell numbers (Fig. 3B), our results support the hypothesis that Arx-deficient progenitors, which fail to generate a large subset of enteroendocrine cells, adopt an alternative $Sst$-expressing cell fate. As seen for $Sst^+$ cells, $Ghrl^+$ mRNA (Fig. 3A) and the number of Ghrelin-producing cells (Fig. 3B) increase in Arx-deficient intestine. However, as a subset of Ghrelin$^+$ cells expresses Arx in wild-type intestine and as Ghrelin is also co-expressed with GLP1 or $Sst$ (Fig. S4), it is impossible to ascertain the cellular mechanisms leading to the increased number of Ghrelin$^+$ cells in Arx-deficient intestine. Interestingly, our tracing data also reveal that a subset of Serotonin-expressing cells (Fig. 5A) also derive from Arx-deficient cells. Based on the absence of Arx/Serotonin co-expression, we postulated that Serotonin$^+$ cells do not normally arise from Arx progenitors. Thus taken together, our findings suggest that Arx-deficient progenitor cells could be reallocated to the Serotonin-producing EC lineage. Given the high number of Serotonin-expressing EC-cells, we hypothesize that the reallocation of some Arx-deficient cells towards the EC lineage is however not sufficient to significantly impact the number of Serotonin-expressing cells (Fig. 3B). In summary, we conclude that the increase in Sst-expressing cell numbers observed in Arx mutants results from the reallocation of progenitor cells to the Sst lineage rather than from the expansion of Arx-independent Sst$^+$ cell precursors.

**Opposing functions of Pax4 and Arx control the specification of GLP1- (L-cells) and Somatostatin- (D-cells) expressing cells**

The consequences of Pax4 loss-of-function on enteroendocrine cell differentiation have previously been reported [15]. However, since the expression of several hormones, including Glp1 and $Sst$, as well as of downstream transcription factors was not addressed, we decided to reinvestigate the phenotype of Pax4 mutants. Firstly, we determined which endocrine subtypes express Pax4. Due to the lack of working anti-Pax4 antibodies, we took advantage of $Pax4^{+/-}$ ($Pax4^{+/LacZ}$) mice, in which the beta-galactosidase gene is inserted within the $Pax4$ locus [16], to label Pax4-expressing cells (beta-gal$^+$). In adult mice, the beta-gal was co-detected with all hormones tested suggesting that Pax4 is expressed in all enteroendocrine subtypes analyzed, including GLP1-, GIP-, CCK-, Serotonin- and Ghrelin-expressing cells (Fig. 5B). Because of the stability of the beta-gal [8], we could not determine whether Pax4 is expressed in progenitors
and/or in their differentiated descendants. However, the absence of beta-gal expression in the villi suggests that Pax4 expression is not maintained in mature endocrine cells (Fig. 5B). Analyses of Pax4 mutant mice revealed that, in contrast to Arx-deficient animals, ChgA expression in P2 small intestine and colon is severely reduced (22.1% ± 1.1 and 41.0% ± 7.1, respectively, as compared to controls; Fig. 6A). Furthermore, the differentiation of many enteroendocrine subtypes is impaired, as demonstrated by the decrease of Nts, Gastrin and Sct expression in the small intestine and the almost absence of Gip and Tph1 expression (Fig. 6A and Table S1). Interestingly, Sst expression, which is increased in Arx mutants, is lost in Pax4-deficient mice. Conversely, Gip1 expression is augmented in Pax4 mutants, whereas it is lost in Arx mutants (compare Fig. 3A to Fig. 6A). In addition, the concomitant increase of Gip1 and Pyy transcripts in Pax4-deficient mice suggests an augmentation of the number of L cells. In agreement with this hypothesis, counting of GLP-1-positive cells in the ileum (P2) revealed a doubling of the number of L-cells (Fig. 6B). Finally, as previously described [21], we

Figure 3. Hormone expression in Arx-deficient intestine. (A) Real time RT-PCR analyses of various intestinal hormones mRNAs in Arx-deficient and control small intestine and colon at 2 days postpartum (n = 5). Glp1, Gip, Cck, Pyy, Nts and Sct mRNA levels are significantly reduced in Arx mutant mice, whereas Sst and Ghrl expression are increased in the small intestine. (B) Quantification of Sst+ and Ghrl+ cells in Arx+/− (n = 3) and Arx−/− P1 duodenum (n = 3). Both Sst and Ghrl-expressing cell numbers increase in Arx-deficient duodenum while the number of Serotonin-cells (5HT) is unchanged. Student’s T-test *p<0.05, **p<0.01, ***p<0.001.

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confirmed an increase in *Ghel* expression in Pax4 mutants. In summary, these results indicate that Pax4, like Arx, is necessary for the proper development of GIP, Gastrin and Neurotensin cells in the small intestine while Serotonin-producing cells are exclusively Pax4-dependent. Furthermore the allocation of GLP1+ and Sst+ lineages appears to be regulated by the opposing roles of Arx and Pax4 respectively.

**Arx** expression is upregulated in **Pax4**-deficient intestine but **Pax4** is unaltered in **Arx** mutant

To determine the position of Arx and Pax4 in the cascade of transcription factors implementing the enteroendocrine differentiation program, we analyzed the expression of a series of TFs in Arx- and Pax4-deficient intestines. The levels of *Neurog3*, *NeuroD1*, *Insom1*, *Rfx6*, *MafB* and *Nkx2.2* transcripts remained unchanged in both knockouts (Fig. 7 and Fig. S5). In contrast, *Foa1* and *Foxa2* expressions were moderately but significantly increased in Arx-deficient small intestine as well as *Pdx1* in the colon (Fig. 7A). As the inactivation of these TFs leads to decreased Sst expression [13,22], their up-regulation in Arx mutant mice could in turn promote Sst transcription. The expression of *Pax6*, which is generally considered to be a late TF in islet cell development, slightly decreased in Pax4 mutants but is unaffected in Arx mutants (Fig. 7). Importantly, we observed 2.1±0.37 times more Arx mRNA in Pax4-deficient small intestines compared to controls (Fig. 7B). However, we could not detect a significant increase in Pax4 expression in Arx mutants (Fig. 7A). To determine whether either Arx or Pax4 was sufficient to induce phenotypic changes in enteroendocrine cells we performed gain of function experiments in STC-1 cell line [23]. In this experimental system, overexpression of Pax4 or Arx did not alter Arx or Pax4 transcription respectively or hormone gene expression (Fig. S6). In summary the expression of many TFs is unaffected in Arx- or Pax4-deficient mice, suggesting that Arx and Pax4 act downstream or in parallel pathways. The strong induction of Arx, both in the small intestine and colon of Pax4 KO mice, suggests that Pax4 controls the specification of endocrine subtypes through the repression of Arx.

**Discussion**

Previous studies have demonstrated the essential role of Arx in cell fate decision during pancreas development and forebrain morphogenesis [17,20,24]. In this study, we showed that Arx and Pax4 are required for the differentiation of several enteroendocrine cells in the small and large intestine and control the specification of endocrine subtypes (Fig. 8). Arx expression is strictly dependent on Neurog3 demonstrating that Arx is
exclusively found in the intestinal endocrine lineage and not in other intestinal cell types. We revealed Arx expression in subsets of post-mitotic Neurog3-positive endocrine progenitor cells in the embryonic and adult intestine. Arx is subsequently maintained in nascent hormone-expressing cells still located in the crypt. These include the Gluco-incretin GLP1- and GIP-expressing cells, which derive from Arx-positive progenitors, but exclude Somatostatin- and Serotonin-expressing cells. Lineage tracing experiments in wild-type mice using BAC transgenics would be required to further ascertain that Somatostatin- and Serotonin-expressing cells do not arise from Arx-expressing progenitors. Mature, Chromogranin A-positive, endocrine cells present in the villi are devoid of Arx. In summary, our results suggest that Arx transcription is switched on in selected endocrine progenitors to control their destiny. Arx is then transiently expressed in early hormone-expressing cells and subsequently switched off. This expression pattern contrasts with the observation made in the pancreas where the Arx protein remains expressed in mature alpha-cells in adult islets [19] and, importantly, suggests that Arx controls the differentiation of enteroendocrine cells but not their function.

Figure 6. Hormone expression in Pax4-deficient intestine. (A) Real time RT-PCR analyses of various intestinal hormones mRNAs in Pax4-deficient and control small intestine and colon at 2 days postpartum (n = 4). Gip, Nts, Gast, Sct and Tph1 mRNA levels decrease significantly in Pax4 mutant small intestine, Glp1 and Ghrl expressions increase in both the small intestine and colon. (B) Quantification of GLP1+ cells in Pax4+/− (n = 3) and Pax4−/− P1 ileum (n = 3). GLP1-expressing cells are more abundant in Pax4 mutant ileum. Student’s T-test *p<0.05, **p<0.01, ***p<0.001.

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While we observed a loss or drastic reduction of several enteroendocrine cell populations in Arx-deficient intestines, Secretin- or Gastrin-expressing cells were only found to be reduced. This suggests that an Arx-independent program can lead to the differentiation of Secretin- or Gastrin-cells. Interestingly, we observed a very significant increase of both Sst mRNA and somatostatin-expressing cell numbers. On the other hand, the expression of ChgA, encoding a component of the secretory granules of most enteroendocrine cells, was found unchanged, suggesting that the total enteroendocrine cell number is unaffected. Taken together, these data indicate that, upon Arx deficiency, a reallocation of developing enteroendocrine progenitor cells towards the somatostatin lineage occurs.

The identification of Somatostatin+/Arx− cells in tracing experiments further supports the notion of a cell type conversion rather than a function of Arx in the repression of the transcription of the Sst gene. Notably, the numbers of Somatostatin-expressing delta-cells (in addition to beta-cells) are also increased in Arx-deficient pancreata, suggesting a similar function of Arx in the repression of the differentiation program leading to the generation of somatostatin-producing cells. It is tempting to speculate that a similar reallocation occurs towards Ghrelin-expressing cells, the latter being also increased in number in Arx KO. However, due to the co-expression of Ghrelin with several other intestinal hormones (GLP-1, Somatostatin), it is unclear whether Ghrelin-expressing cells do correspond to a distinct enteroendocrine subtype. Interestingly, Ghrelin+ cell numbers also increase in Nkx2.2- or Pax4-deficient embryonic intestines [14,21], two genes that have been suggested to directly regulate ghrelin expression [21,25]. However, in the current study the increase in the number of Ghrelin+ cells does not result from a down-regulation of Nkx2.2 or Pax4, since mRNA levels of both genes do not change in the small intestine and colon of newborn Arx-deficient mice. Since a co-detection of Glucagon and Ghrelin is frequent in immature developing alpha-cells in the embryonic pancreas [26], another possibility could be that Ghrelin+ cells might correspond to L-cells precursors (GLP1-negative) blocked in their differentiation.

**Figure 7. Expression of transcription factors in Arx- and Pax4-deficient intestines.** Real time PCR analyses in (A) Arx- and (B) Pax4- (n = 4) deficient (n = 5) and control (n = 5) small intestine and colon at 2 days postpartum. (A) Pdx1 and Foxa1/a2 expression are increased in Arx mutant colon and small intestine, respectively. (B) Arx is significantly upregulated in Pax4 mutants. Student’s T-test *p<0.05, **p<0.01, ***p<0.001.

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While we observed a loss or drastic reduction of several enteroendocrine cell populations in Arx-deficient intestines, Secretin- or Gastrin-expressing cells were only found to be reduced. This suggests that an Arx-independent program can lead to the differentiation of Secretin- or Gastrin-cells. Interestingly, we observed a very significant increase of both Sst mRNA and somatostatin-expressing cell numbers. On the other hand, the expression of ChgA, encoding a component of the secretory granules of most enteroendocrine cells, was found unchanged, suggesting that the total enteroendocrine cell number is unaffected. Taken together, these data indicate that, upon Arx deficiency, a reallocation of developing enteroendocrine progenitor cells towards the somatostatin lineage occurs.

The identification of Somatostatin+/Arx− cells in tracing experiments further supports the notion of a cell type conversion rather than a function of Arx in the repression of the transcription of the Sst gene. Notably, the numbers of Somatostatin-expressing delta-cells (in addition to beta-cells) are also increased in Arx-deficient pancreata, suggesting a similar function of Arx in the repression of the differentiation program leading to the generation of somatostatin-producing cells. It is tempting to speculate that a similar reallocation occurs towards Ghrelin-expressing cells, the latter being also increased in number in Arx KO. However, due to the co-expression of Ghrelin with several other intestinal hormones (GLP-1, Somatostatin), it is unclear whether Ghrelin-expressing cells do correspond to a distinct enteroendocrine subtype. Interestingly, Ghrelin+ cell numbers also increase in Nkx2.2- or Pax4-deficient embryonic intestines [14,21], two genes that have been suggested to directly regulate ghrelin expression [21,25]. However, in the current study the increase in the number of Ghrelin+ cells does not result from a down-regulation of Nkx2.2 or Pax4, since mRNA levels of both genes do not change in the small intestine and colon of newborn Arx-deficient mice. Since a co-detection of Glucagon and Ghrelin is frequent in immature developing alpha-cells in the embryonic pancreas [26], another possibility could be that Ghrelin+ cells might correspond to L-cells precursors (GLP1-negative) blocked in their differentiation.
Arx and Pax4 have been demonstrated to have antagonistic functions during the specification of islet sub-types in the pancreas. We therefore postulated that similar mechanisms could operate in the intestine. We found that in the intestine, the different enteroendocrine cell types similarly and differentially require Arx and Pax4. Indeed, Gip, Cck, Set, Gast, and Nts intestinal expression are reduced in both knockout mouse, suggesting that the differentiation of these progenitors into GLP1-expressing cells is impaired. Sst- and Serotonin (5-HT)-expressing cells while the differentiation of Sst-, Serotonin (5-HT)-, Gast-, Gip- and Nts-expressing cells is impaired. Key transcription factors controlling intestinal cell destiny are also indicated.

**Figure 8. Model of enteroendocrine subtype specification during small intestine development: roles of Arx and Pax4.**

Gast-, Gip-, Nts-, Set-, CCK- and GLP1-expressing cells arise from endocrine progenitors expressing Neurog3 then Pax4 and Arx. Upon Arx inactivation these progenitors are reallocated into Sst-expressing cells while the differentiation of Gast-, Gip-, Nts-, Set-, CCK- and GLP1-expressing progenitors is impaired. Sst- and Serotonin (5-HT)-expressing cells are generated from progenitors expressing Neurog3 then Pax4. Inactivation of Pax4 leads to the up-regulation of Arx and the differentiation of these progenitors into GLP1-expressing cells, while the differentiation of Sst-, Serotonin (5-HT)-, Gast-, Gip- and Nts-expressing cells is impaired. Key transcription factors controlling intestinal cell destiny are also indicated.

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In conclusion, our study reveals that Arx and Pax4 are similarly and differentially required for enteroendocrine cell differentiation downstream of the proendoctrine transcription factor Neurog3 by controlling subtype specification and number. Our results also provide evidence that Arx and Pax4 antagonistically regulate L- and D-cell fate specification, respectively. Arx represses Foxa1/ Foxa2 while Pax4 represses Arx. In humans, Aristaless-related homeobox gene (ARX) mutation leads to several neurological disorders. Other reported symptoms can include severe growth retardation, abnormal genitalia, disregulation of glycemia and intractable diarrhea [24,30,31,32]. We propose that impaired enteroendocrine cell differentiation may be the cause of the chronic diarrhea in ARX-deficient patients as others and we reported similar phenotype in mice and patients with a mutation in Neurog3 and lacking enteroendocrine cells [3,33]. Finally, considering the key role of enteroendocrine cells and hormones in nutrient sensing, food intake and glucose homeostasis, it would
potentially be of interest in the future to find means to modulate the ratio of various enteroendocrine cell types in the adult mouse intestine and determine the consequences on energy metabolism. Such studies could stimulate novel therapeutic strategies to treat metabolic disorder, such as obesity and type-2 diabetes.

**Materials and Methods**

**Animals**

Animal experiments were supervised by G. Gradwohl (agreement No C67-59 approved by the direction des Services Vétérinaires, Strasbourg, France) in compliance with the European legislation on care and use of laboratory animals. Pax4 and Arx null mice were described previously [16,17]. Embryos were considered to be at E0.5 day of gestation at noon of the day the vaginal plugs were detected. Animals of both sexes were analyzed except in Fig. 3A were only females were studied. Unless otherwise indicated adult mice were analyzed at 3–6 months of age. Arx and Pax4 heterozygous mice are kept on a 129/Sv background and Arx or Pax-deficient mice and littermates analyzed at P1–2. Expression studies described in Figure 1 and 2 were performed on CD1 mice.

**Immunohistochemistry and in situ hybridization**

Tissues were fixed in 4% PFA, o/n at 4°C, washed in PBS, equilibrated in 20% sucrose at 4°C and embedded in OCT compound. The following primary antibodies were used: guinea pig anti-Neurog3 at 1:1000 (provided by M. Sander, University of California - San Diego, La Jolla, CA, USA), chicken anti-beta-galactosidase at 1:5000 (Abcam), rabbit anti-Arx at 1:500, goat anti-Chga at 1:200 (Santa Cruz), goat anti-GLP1 at 1:100 (Santa Cruz), rabbit anti-GLP1 at 1:500 (Phoenix), goat anti-GIP at 1:100 (Santa Cruz), rabbit anti-GIP at 1:500 (Phoenix), goat anti-CCK at 1:500 (Santa Cruz), rabbit anti-CCK/Gastrin at 1:750 (provided by C. Roche, Inserm U865 Lyon, France), goat anti-Gastrin at 1:50 (Santa Cruz), goat anti-Somatostatin at 1:200 (Santa Cruz), mouse anti-Ghrl at 1:1500 (Catherine Tomasetto, IGBMC, Strasbourg, France), rabbit anti-Ghrl at 1:1000, rabbit anti-Serotonin at 1:1000 (Diasorin Incstar), rabbit anti-Neurotensin at 1:500 (Phoenix) and rabbit anti-PYY at 1:500 (Phoenix). Secondary antibodies conjugated to DyLight-488, DyLight-549 and DyLight-649 (Jackson ImmunoResearch Laboratories) were used at 1:500. For anti-Arx and anti-beta-galactosidase staining, signal amplification was performed using biotin anti-rabbit or anti-chicken coupled antibody and streptavidin-conjugated to DyLight-488, DyLight-549 and DyLight-649 (Jackson ImmunoResearch Laboratories) were used at 1:500. For anti-Arx staining, signal amplification was performed using biotin anti-rabbit or anti-chicken coupled antibody and streptavidin-conjugated to DyLight-488, DyLight-549 and DyLight-649 (Jackson ImmunoResearch Laboratories) were used at 1:500. For secondary antibodies the primary antibodies were used: guinea pig anti-Neurog3 at 1:1000 (provided by M. Sander, University of California - San Diego, La Jolla, CA, USA), chicken anti-beta-galactosidase at 1:5000 (Abcam), rabbit anti-Arx at 1:500, goat anti-Chga at 1:200 (Santa Cruz), goat anti-GLP1 at 1:100 (Santa Cruz), rabbit anti-GLP1 at 1:500 (Phoenix), goat anti-GIP at 1:100 (Santa Cruz), rabbit anti-GIP at 1:500 (Phoenix), goat anti-CCK at 1:500 (Santa Cruz), rabbit anti-CCK/Gastrin at 1:750 (provided by C. Roche, Inserm U865 Lyon, France), goat anti-Gastrin at 1:50 (Santa Cruz), goat anti-Somatostatin at 1:200 (Santa Cruz), mouse anti-Ghrl at 1:1500 (Catherine Tomasetto, IGBMC, Strasbourg, France), rabbit anti-Ghrl at 1:1000, rabbit anti-Serotonin at 1:1000 (Diasorin Incstar), rabbit anti-Neurotensin at 1:500 (Phoenix) and rabbit anti-PYY at 1:500 (Phoenix). Secondary antibodies conjugated to DyLight-488, DyLight-549 and DyLight-649 (Jackson ImmunoResearch Laboratories) were used at 1:500. For anti-Arx and anti-beta-galactosidase staining, signal amplification was performed using biotin anti-rabbit or anti-chicken coupled antibody at 1:500 (Jackson ImmunoResearch Laboratories) and streptavidin-Cy3 conjugate at 1:500 (Molecular Probes). Nuclei were stained with DAPI and slides were mounted in Aqua- Poly/Mount (Polysciences).

**Real time PCR analysis**

Total RNA from the whole small intestine (duodenum, jejunum and ileum) and colon was extracted using TRIzol Reagent (Invitrogen). Reverse transcription was performed using Transcriptase Reverse Transcriptase (Roche). Quantitative PCRs were performed using mouse-specific TaqMan primers and probes (Applied Biosystems) recognizing Neurog3 (Mm00437606_s1), Chga (Mm00134341_m1), Arx (Mm00545903_m1), Pax4 (Mm01159-036_m1), Pax6 (Mm0043081_m1), Pdx1 (Mm00435565_m1), Foxa1 (Mm00484713_m1), Foxa2 (Mm00839704_mH), Ins1 (Mm02381292_s1), Rf56 (Mm00624115_m1), Neurod1 (Mm0128-0117_m1), Mafb (Mm00627481_s1), Pyy (Mm00520715_m1), Ns (Mm00481140_m1), Cck (Mm00464730_m1), Sat (Mm00441-235_g1), Gip (Mm00433601_m1), Gcg/Glp1 (Mm00801712_m1), Tph1 (Mm00493794_m1), Gast (Mm00772211_g1), Sst (Mm004356671_m1), Ghrl (Mm00445430_m1), Muc2 (Mm00458299_m1), Gf1 (Mm00515835_m1) or UPL probes #20 (Roche) for Nkx2.2 (5’ primer gcagcgacaacccctaca, 3’ primer atttggagctcgagtcttgg) with TaqMan Light Cycler 480 Probes Master Mix (Roche) on Light Cycler 480 (Roche). Gene expression levels were normalized to β-actin (4352933E).

**Morphometric analysis**

Somatostatin+/ cells and Ghrelin+ cells were counted after immunostaining on approximately 60 sections of the duodenum at P1 and 3 Arx+/ and 3 Arx− samples. For GLP1+ cells, approximately 30 sections of the ileum at P1 were counted, on 3 Pax4+/ and 3 Pax4− samples. The numbers of hormone+ cells were normalized according to the area of the sections estimated by the surface of DAPI staining.

**Gain of function studies in STC-1 cells**

STC-1 cells were transfected with 2 µg of pCAG-Arx-ires-b-gal, pCAG-Pax4-ires-b-gal and pCAG-GFP as control. 48 h after transfection Arx, Pax4 and hormones mRNA levels were quantified by RT-qPCR as described above and normalized to Rpdp0 (TaqMan assay Rpdp0; Mm01974747_gH).

**Statistics**

Values are presented as mean ± SD. P values were determined using the 2-tailed Student’s t test with unequal variance. P values of less than 0.05 were considered significant. ***, p<0.001, **, p<0.01, *, p<0.05.

**Supporting Information**

**Figure S1 Arx-expressing cells are located in the intestinal crypts in the adult mouse intestine.** Intestinal sections were stained with an anti-Arx antibody. Red arrows point to Arx-positive cells. (TIF)

**Figure S2 Arx is expressed in post-mitotic cells in intestinal crypts.** Sections of adult mouse small intestine were stained with an anti-Arx antibody (revealed in red) and an anti-Ki-67 antibody (revealed in green). A representative image of an Arx-positive/Ki-67-negative nucleus found in the small intestine crypt compartment is shown. (TIF)

**Figure S3 GLP1, GIP, CCK, Gastrin, Nts and PYY cells are lost in Arx-deficient mice.** Immunostaining of wild-type and P2 Arx-mutant mice (small intestine sections) using antibodies against intestinal peptides and serotonin. Hormone+ cells are green. (TIF)

**Figure S4 Ghrl is detected in some GLP1+ cells and Sat+ cells.** Co-immunostaining of Ghrl and GLP1 or Sst on intestinal sections of wild-type adult mice. Yellow arrows point to co-expressing cells. (TIF)

**Figure S5 Expression of Neurog3, Neurod1, Rfx6, Mafb and Nkx2.2 mRNAs is not affected in Arx- or Pax4-deficient small intestine.** Quantification of mRNAs encoding key endocrine transcription factors in Arx- and Pax4-deficient small intestine. Real time PCR analysis in Arx (n=5) and Pax4-deficient mice (n=4) and control small intestine and colon, 2 days after birth. (TIF)
Figure S6  Arx and Pax4 over-expression (OE) in STC-1 enteroendocrine cell line. STC-1 cells were transfected with plasmids expressing Pax4, Arx or GFP under the control of the CAG (Cytoamegalovirus enhancer/chicken β-actin) promoter. 48 h after transfection overexpression of Arx and Pax4 was measured by mRNA quantification in Arx (A) and Pax4 (B) transfected cells (upper panels). A 1500- and 400-fold increase of Arx or Pax4 was observed after transfection with Arx or Pax4 –expression plasmids respectively when compared to GFP-transfected STC-1 cells. (C) The expression of mRNAs encoding enteroendocrine hormones did not show significant variation upon Arx or Pax4 OE suggesting that neither Arx nor Pax4 is able to promote endocrine differentiation or hormone gene transactivation in STC-1 cells. Tph1 mRNA, encoding Tryptophan hydroxylase 1 the rate-limiting enzyme in Serotonin synthesis, was used to evaluate the induction of Serotonin producing cells. Values represent means of fold changes (Arx-transfected/GFP-transfected or Pax4-transfected/GFP-transfected) of 3 independent experiments ± SD. (TIF)

Table S1  Hormone mRNA levels in the small intestine and colon of Arx- and Pax4-deficient mice at P2. Table summarizing RT-qPCRs data presented in figure 3 and 6. Results are compared to controls and expressed in fold change. Tph1 mRNA, endcoding Tryptophan hydroxylase 1 the rate-limiting enzyme in Serotonin synthesis, was used to evaluate Serotonin producing cells. n = 4–5 for mutants and controls, Student's T-test *p<0.05, **p<0.01, ***p<0.001. (TIF)

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Author Contributions

Conceived and designed the experiments: AB PC GG. Performed the experiments: AB CC EG MC AM. Analyzed the data: AB EG MC GG PC. Wrote the paper: AB PC GG.

References

1. Date Y, Kojima M, Hosoda H, Sashoguchi A, Mondal MS, et al. (2000) Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141: 4253–4261.
2. Sakata I, Nakamura K, Yamazaki M, Matsubara M, Hayashi Y, et al. (2002) Gastrin-producing cells exist as two types of cells, closed- and opened-type, in the rat gastrointestinal tract. Peptides 23: 531–536.
3. Drucker DJ (2007) The role of gut hormones in glucose homeostasis. J Clin Invest 117: 24–32.
4. Murphy KG, Bloom SR (2006) Gut hormones and the regulation of energy homeostasis. Nature 444: 854–859.
5. Mellitzer G, Buecher A, Lobstein V, Michel P, Robine S, et al. (2010) Loss of enteroendocrine cells in mice alterts lipid absorption and glucose homeostasis and impairs postnatal survival. J Clin Invest 129: 1708–1721.
6. Shirove NF, Helmraith MA, Wang YY, Amalaffi B, Henning SJ, et al. (2007) Intestine-specific ablation of mousen atonal homolog 1 [Math1] reveals a role in cellular homeostasis. Gastroenterology 132: 2478–2486.
7. Yang Q, Berrinham NA, Fongelder MJ, Zoghbi HY (2001) Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science 294: 2153–2158.
8. Jenny M, Uh C, Roche C, Dalai I, Guillermín V, et al. (2002) Neurogenin3 is differentially required for enteroendocrine cell fate specification in the intestinal and gastric epithelium. Embo J 21: 6338–6347.
9. Lee CS, Perreau N, Bresteil JE, Kaestner HK (2002) Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity. Genes and Development 16: 1465–1474.
10. Gradwohl G, Dierich A, LeMeur M, Guillemot F (2000) Neurogenin3 is required for the development of the four enteroendocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A 97: 1607–1611.
11. Gierl MS, Karousias N, Wende H, Strehle M, Birchmeier C (2006) The zinc-finger factor Euxin, [Euxin] is essential for the development of pancreatic beta cells and intestinal endocrine cells. Genes and Development 20: 2465–2478.
12. Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, et al. (1997) Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in Beta2/neuroD-deficient mice. Genes Dev 11: 2323–2334.
13. Ye DZ, Kaestner HK (2009) Foxa1 and Foxa2 control the differentiation of goblet and enteroendocrine L- and D-cells in mice. Gastroenterology 137: 2052–2062.
14. Desai S, Lesonis Z, Pagh-Bernard A, Schrunk J, Doyle MJ, et al. (2000) Nkx2.2 regulates cell fate choice in the enteroendocrine cell lineages of the intestine. Dev Biol 231: 58–66.
15. Larson LL, Se-Cong L, Hougaard DM, Sosa-Pineda B, Gruss P (1998) Pax 4 and 6 regulate gastrointestinal endocrine development. Mech Dev 79: 153–158.
16. Sosa-Pineda B, Chowdhury K, Torres M, Oliver G, Gruss P (1997) The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature 386: 399–402.
17. Colombat P, Mansouri A, Hecksher-Sorensen J, Serup P, Kroll J, et al. (2003) Opposing actions of Arx and Pax4 in endocrine pancreas development. Genes and Development 17: 2591–2603.
18. Colombat P, Hecksher-Sorensen J, Kroll J, Berger J, Kiedel D, et al. (2007) Embryonic entoendocrine pancreas and mature beta cells acquire alpha and PP cell phenotypes upon Arx misexpression. J Clin Invest 117: 961–970.