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GATA factors regulate proliferation, differentiation, and gene expression in small intestine of mature mice

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Abstract

Background & Aims—GATA transcription factors regulate genes in multiple organs to control proliferation and differentiation. GATA4 is expressed in the proximal 85% of the small intestine, where it regulates the expression of genes that are specifically expressed by absorptive enterocytes. GATA6 is co-expressed with GATA4 but is also expressed in the ileum; its function in the mature small intestine is unknown.

Methods—We investigated the function of GATA6 in small intestine using adult mice with inducible disruption of Gata6, or Gata6 and Gata4, specifically in the intestine.

Results—In ileum, deletion of Gata6 reduced proliferation and numbers of enteroendocrine cells, increased numbers of goblet-like cells in crypts, caused loss of Paneth cells, and altered

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expression of genes specific to absorptive enterocytes. In contrast, in jejunum and duodenum, deletion of Gata6 increased numbers of Paneth cells. Deletion of Gata6 and Gata4 resulted jejunal and duodenal phenotype that was nearly identical to that in the ileum after deletion of Gata6 alone, demonstrating that most GATA4 functions are redundant with those of GATA6.

Conclusion—GATA transcription factors are required for proliferation, secretory cell differentiation, and expression of genes by absorptive enterocytes in the small intestinal epithelium.

Keywords

GI development; gene regulation; transgenic mouse; secretion

The mature mammalian small intestine is a highly regenerative organ in which the orderly differentiation of cells along the crypt-villus axis, and the precise distribution of specialized cell types and expression of proteins are essential for intestinal function. Stem cells located at or near the base of crypts produce transit-amplifying cells that undergo a series of cell fate decisions ultimately giving rise to four main cell types. Absorptive enterocytes, the most numerous villus cell type, express digestive enzymes and transporters in a tightly regulated spatial pattern designed for optimal digestion and absorption of nutrients. Mucus-secreting goblet cells and defensin-secreting Paneth cells, are necessary for maintaining a dynamic mucosal defensive barrier, and enteroendocrine cell subpopulations display a functional diversity characterized by the regional segregation of hormone secretions that activate or repress gastrointestinal processes. Absorptive enterocytes, goblet cells, and enteroendocrine cells migrate up to populate the villus epithelium and turn over in three to four days, whereas Paneth cells migrate to the base of crypts and turn over at a slower rate of three to six weeks.¹

Wnt and Notch signaling play essential roles in the regeneration of the intestinal epithelium. Disruption of Wnt signaling results in a complete loss of proliferation and a decrease in secretory cell differentiation²⁻⁶ whereas over-activation of Wnt signaling leads to hyperproliferation, enlarged crypts, stunted villi, and an increase in secretory cells.⁷ Disruption of Notch signaling results in a decrease in crypt cell proliferation and an excessive number of secretory cells,⁸⁻¹¹ whereas over-activation of Notch signaling results in an increase in cell proliferation and a reduction of all secretory cell types.¹² Notch signaling regulates the balance of absorptive vs. secretory cells by activating hairy and enhancer of split 1 (HES1),¹³ a basic helix-loop-helix transcription factor that selects the absorptive enterocyte lineage. Progenitor cells that escape Notch signaling and activation of *Hes1* gene transcription express atonal homolog 1 (ATOH1), a basic helix-loop-helix transcription factor that selects the secretory cells (i.e., enteroendocrine, goblet, and Paneth cells).¹⁴ ATOH1-positive secretory progenitors then undergo a series of decisions ultimately resulting in a tightly regulated distribution and localization of mature secretory cells.

GATA proteins are conserved transcription factors that regulate proliferation, differentiation, and gene expression in multiple organs.¹⁵ GATA4 is expressed in the crypt and villus epithelium in the proximal 85% of adult small intestine but is absent from distal ileum,¹⁶⁻¹⁹ whereas GATA6 is expressed in the crypt and villus epithelium throughout the small intestine, including distal ileum.^{16-18, 20} Deletion or mutation of GATA4 results in a decrease in the expression of absorptive enterocyte genes normally found in jejunum, and an increase in the expression of ileal genes demonstrating that GATA4 mediates jejunal-ileal identities in absorptive enterocyte gene expression and function.^{16, 21, 22} Expression of a dominant-negative GATA4 mutant produces not only the jejunal-ileal changes in gene expression, but also alterations in enteroendocrine and goblet cells,¹⁶ suggesting a role for GATA factors in secretory cell differentiation. The function of GATA6 in the adult small

intestine is unknown. The hypothesis to be tested in this study is that GATA factors are necessary for secretory cell differentiation, and possibly other functions, in the small intestine.

Materials and Methods

Mice

Previously established and confirmed *Gata6^{loxP/loxP}*, *Gata4^{flap/flap}* and transgenic Villin^{CreER}^{T2} mice^{16, 20, 22, 23} were used in this study to produce conditional, inducible deletion of *Gata6* or both *Gata6* and *Gata4* in the intestinal epithelium. *Gata6^{loxP/loxP}*, Villin^{CreER}^{T2}-positive (*G6del*), *Gata6^{loxP/loxP}*, *Gata4^{flap/flap}*, Villin^{CreER}^{T2}-positive (*G6G4del*), and *Gata6^{loxP/loxP}*, Villin^{CreER}^{T2}-negative or *Gata6^{Wt/Wt}*, Villin^{CreER}^{T2}-positive (*Control*) mice, 6-8 wk of age, were treated with tamoxifen as described.^{16, 22} Tissue was collected 28 days after treatment, unless otherwise indicated. In a subset of male mice, food intake was determined by measuring consumption during the last 3 days before terminal tissue collection. After the 3 day food intake analysis, body weight was measured, the mice were killed, and blood and tissues were collected. From serum, cholesterol and triglyceride levels were determined. Approval was obtained from the Institutional Animal Care and Use Committee.

Tissue isolation

Mice were dissected as previously described.¹⁶ Samples of ileum were taken from distal small intestine adjacent to the ileocecal valve, samples of jejunum from the geometric center of the small intestine, and samples of duodenum from the first centimeter adjacent to the pylorus. In selected mice, bromodeoxyuridine (BrdU) (0.1 ml of 10 mg/ml) was injected two hours prior to dissection.

Immunohistochemistry and electron microscopy

Immunostaining of intestinal segments was conducted as previously described¹⁶ (see supplementary expanded Materials and Methods for a list of antibodies). For electron microscopy (EM), intestinal segments were fixed in 1.25% glutaraldehyde, 4% formaldehyde, 0.1M cacodylic buffer, pH 7.4 at 4°C overnight. EM was conducted in the Harvard Digestive Disease Center imaging core at Beth Israel Deaconess Medical Center.

Villus and crypt measurements and cell counting

Villus length and crypt depth were measured using image J software (<http://rsb.info.nih.gov/ij/>). The total number of villus and crypt cells was determined by counting the visible nuclei in the epithelial layer. The total number of alcian blue-positive cells on villi was determined as a percentage of total villus epithelial cells. The total number of alcian blue-, Ki67-, BrdU-, or lysozyme (LYZ)-positive cells in crypts was determined as total number per crypt, and the average number of chromogranin A (CHGA)-positive cells was expressed as a fraction of total epithelial cells (villi and crypts) from a minimum of 5000 epithelial cells counted. For all determinations (blinded and conducted on a minimum of 5 animals per group, unless otherwise indicated), a minimum of six villi or six crypts per slide were analyzed.

RNA isolation and gene expression analysis

RNA was isolated from 0.5 to 1.0 cm intestinal segments, and gene expression was determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) as previously described^{16, 22, 24, 25} using validated primer pairs (Supplementary Figure 1). Ileal messenger RNA (mRNA) was also analyzed by whole-genome gene expression analysis

using the Affymetrix Mouse Gene 1.0 ST array by the molecular genetics core facility at Children's Hospital Boston. A minimum of 5 mice in each group were analyzed by qRT-PCR unless indicated otherwise; 3 mice in each group were analyzed by microarray.

Statistical analyses

Data are expressed as mean $\pm$ SD. Statistically significant differences were determined by the two-tailed Student's *t* test. Differences were considered statistically significant at $P < .05$.

Results

Intestinal *Gata6* deletion results in a reduction in villus length, villus epithelial cell number and crypt proliferation in ileum

GATA6 was expressed in all differentiated and proliferating cells in the mature mouse small intestinal epithelium with the highest staining intensity in the proliferative crypt compartment (Supplementary Figure 2). To determine the function of GATA6 in this tissue, a tamoxifen-inducible, intestine-specific *Gata6* deletion model (*G6del*) was established (Supplementary Figure 3). Mice sacrificed up to 4 weeks after *Gata6* deletion exhibited normal growth and activity; in a cohort of male mice, body weights and plasma cholesterol and triglyceride levels were not different from controls (data not shown). However, food intake measured during the 3 days prior to terminal dissection was $>30\%$ lower for *G6del* mice as compared to controls ($P < .01$).

Initial studies focused on distal ileum, where GATA6 is normally expressed, but GATA4 is not. Inducible deletion of *Gata6* in adult mice resulted in villi that were 27% shorter and contained 29% fewer epithelial cells as compared to *Controls*, whereas the depth and cell number of crypts remained unchanged (Figure 1A). Cleaved caspase 3 immunostaining was not different between *G6del* and *Control* ileum (data not shown) indicating that apoptosis is not influenced by *Gata6* deletion. However, the number of Ki67-positive (Figure 1B) and BrdU-positive cells (Figure 1C) was reduced, indicating that *Gata6* deletion results in a decrease in crypt cell proliferation.

Intestinal *Gata6* deletion results in an altered allocation and differentiation of secretory cells in ileum

Microarray analysis conducted on samples of ileum collected 4 weeks after *Gata6* deletion showed a shift in the expression of secretory cell marker transcripts in which enteroendocrine and Paneth cell markers were down-regulated, and goblet cell markers were up-regulated (Supplementary Figure 4A). The decrease in enteroendocrine markers was confirmed by qRT-PCR for *Chga*, glucagon (*Gcg*) and peptide YY (*Pyy*), and by a decrease in the number of CHGA-positive cells ($P < .05$) (Figure 2A). These data suggest that conditional *Gata6* deletion causes a decrease in enteroendocrine cell allocation in the ileum.

The increase in goblet cell markers in *G6del* ileum (Supplementary Figure 4A) was supported by an increase in the number of alcian blue-positive cells ($P < .001$, Figure 2B). Interestingly, the number of alcian blue positive cells on villi was not altered. The alcian blue-positive crypt cells expressed mucin 2 (MUC2), but not trefoil factor 3 (TFF3) (Figure 2C). In agreement, *Muc2* mRNA abundance was increased 1.6-fold ($P < .05$), whereas that of *Tff3* remained unchanged (Figure 2D). These data indicate that conditional *Gata6* deletion does not alter goblet cell differentiation and migration onto villi, but causes an accumulation of goblet-like cells in crypts.

After 4 weeks of *Gata6* deletion, immunostaining for the Paneth cell marker LYZ was decreased in crypts and overlapped with alcian blue-positive cells (Figure 2C). The number

of LYZ-positive cells and *Lyz* mRNA abundance were reduced by 79% ($P<.001$), and 92% ($P<.001$), respectively (Figure 2E). The decline in *Lyz* mRNA occurred more than 2 weeks after tamoxifen (Supplementary Figure 5A), coinciding with a decrease in typical Paneth cells and an increase in goblet-like cells in crypts (Supplementary Figure 5B). Structural analyses by transmission EM demonstrated that Paneth cells with typical electron dense granules were replaced by cells containing characteristic mucin granules (Figure 2C). These data suggest that Paneth cells fail to properly differentiate and default to a goblet-like cell phenotype after *Gata6* deletion.

In a small subpopulation of the goblet-like cells in crypts of *G6del* ileum (~1 cell per crypt), MUC2 co-localized with alpha-defensin related sequence (DEFA-RS) (Figure 2C), demonstrating co-expression of goblet and Paneth cell markers. Transmission EM revealed structures in *G6del* ileum that contain granules with an electron dense core typical of Paneth cells surrounded by a lighter, granular mass typical of goblet cells (open arrowhead, Figure 2F). These granules, clearly different from 'mixed' granules in nascent intermediate or granular goblet cells described previously,²⁶ were never seen in *Control* ileum, and were likely the granules that co-express goblet and Paneth markers.

Intestinal *Gata6* deletion results in changes in the expression of crypt Wnt targets in ileum

Nuclear β -catenin, the hallmark of active Wnt signaling within cells, was readily detected in Paneth cells at the base of crypts in *Control* ileum, but was markedly reduced in the goblet-like cells at the base of crypts in *G6del* ileum (Figure 3A). The mRNA abundance of the Paneth-specific Wnt target alpha-defensin 1 (*Defa1*) and the Paneth-specific Wnt receptor frizzled 5 (*Fzd5*)²⁷ were down-regulated in the *G6del* ileum, but the transcript abundance of β -catenin and multiple crypt Wnt targets, including myelocytomatosis oncogene (*c-Myc*), CD44 antigen (*Cd44*), leucine rich repeat containing G protein coupled receptor 5 (*Lgr5*), eph receptor B2 (*Ephb2*), *Ephb3*, and *Sox9*, were either unchanged or up-regulated (Figure 3B). Interestingly, EPHB3, a Wnt target expressed in the crypt base region and necessary for Paneth cell localization,²⁸ was expressed in the goblet-like cells at the base of crypts (Figure 3A). Also, SOX9, a Wnt target expressed in Paneth progenitors and required for Paneth cell differentiation,^{29, 30} was up-regulated in the goblet-like cells in the crypts of *G6del* ileum (Figure 3B). These data indicate that conditional *Gata6* deletion does not compromise crypt Wnt signaling in general, and that the goblet-like cells that accumulate in crypts of *G6del* ileum express Wnt targets normally found in Paneth cells and their progenitors (e. g., EPHB3 and SOX9).

Intestinal *Gata6* deletion results in alterations in Notch signaling in ileum

Notch1 or *Hes1* transcript abundance was not different from *Controls*, but *Atoh1* mRNA was up-regulated (Supplementary Figure 4A), suggesting an alteration in secretory cell differentiation. The mRNA abundance of neurogenin 3 (NGN3) that selects enteroendocrine cells in ATOH1-positive progenitors³¹ was down-regulated, whereas that of growth factor independent 1 (GFI1) that promotes goblet/Paneth lineage differentiation at the expense of enteroendocrine cells,³² remained unchanged (Figure 4A). The mRNA abundance of SAM pointed domain containing ets transcription factor (SPDEF), a downstream target of GFI1³³ and also a Wnt target,³⁴ that promotes the goblet cell lineage, was up-regulated, whereas that of the Notch ligand delta-like 1 (DLL1) was down-regulated (Figure 4A). The up-regulation of SPDEF was localized to the goblet-like cells in crypts (Figure 4B). In intestine from *Gfi1*^{-/-}³² and *Spdef*^{KO}³⁴ mice, and from crypts of conditional transgenic *Spdef* over-expressing mice (*Spdef*^{TG}),³³ *Gata6* mRNA levels were not changed (Figure 4C). These data indicate that conditional *Gata6* deletion produces changes in Notch signaling consistent with expansion of the goblet cell lineage, and that *Gata6* is not regulated by GFI1 or SPDEF.

Intestinal *Gata6* deletion results in changes in the expression of specific absorptive enterocyte genes in ileum

In *G6del* ileum, absorptive enterocytes appeared structurally characteristic (EM, data not shown), and expression of the apical sodium dependent bile acid transporter (*Asbt*), a terminal differentiation marker in ileum, was not altered (Figure 5A), indicating that absorptive enterocyte differentiation is generally normal. However, some genes normally expressed at low or undetectable levels in small intestine, many of which encode proteins normally expressed in colon, were up-regulated (e. g. carbonic anhydrase (*Car*) 1 and 2, and claudin 8), while other genes normally expressed in absorptive enterocytes, many of which encode proteins involved in lipid metabolism, were down-regulated (e. g. apolipoprotein C-III, apolipoprotein A-I (*Apoa1*), and fatty acid binding protein 6) (Supplementary Figure 4B). In agreement with the latter observation, functional analyses using the gene ontology (GO) database indicated that lipid metabolism is likely to be affected by conditional *Gata6* deletion (Supplementary Figure 4C). Changes in transcript abundance were confirmed for *Car1* (up-regulated) and *Apoa1* (down-regulated) by qRT-PCR (Figure 5A). The up-regulation of CAR1, which is not normally expressed in ileum, was localized to absorptive enterocytes on villi (Figure 5B).

Intestinal *Gata6* deletion results in an increase in Paneth cells in proximal small intestine

All of the analyses described thus far were performed on ileum where GATA6 is normally expressed, but GATA4 is not.^{16, 19} In jejunum and duodenum, where GATA6 and GATA4 are normally co-expressed,¹⁶ *Gata6* deletion did not alter the number of Ki67-positive proliferating, CHGA-positive enteroendocrine, or alcian blue-positive goblet cells, nor did it alter the mRNA levels of the absorptive enterocyte genes *Car1* and *Apoa1* (see later). *Gata6* deletion also did not alter the expression of *Gata4* or its targets lactase-phlorizin hydrolase and *Asbt*^{16, 22} (data not shown). However, in contrast to the *G6del* ileum where Paneth cells fail to differentiate, the mRNA abundance of multiple Paneth markers (Figure 6A and Supplementary Figure 6A), as well as the immunostaining intensity of LYZ (Figure 6B and Supplementary Figure 6B) all increased in jejunum and duodenum of *G6del* mice. Structural analyses by EM confirmed an increase in the number of cells containing electron dense Paneth-like granules in the crypts of *G6del* jejunum, but also revealed granules that were more variable in size and more widely dispersed in the cell than normal (Figure 6C). Together, these data indicate that the alterations in proliferation, differentiation, and gene expression that occur in the ileum of *G6del* mice do not occur in the proximal small intestine of these mice, but instead reveal an increase in Paneth cells with atypical granules.

Intestinal *Gata6/Gata4* deletion results in changes in proliferation, differentiation and gene expression throughout the small intestine

Conditional deletion of both *Gata6* and *Gata4* (confirmed by qRT-PCR and immunostaining; data not shown) resulted in a phenotype throughout the small intestine that was similar to that found in the *G6del* ileum: fewer Ki67-positive proliferating cells (Figure 7A and B, Supplementary Figure 7A); a decrease in the mRNA abundance of multiple enteroendocrine cell markers and *Ngn3* (Figure 7C, Supplementary Figure 7B, and data not shown), more alcian blue- (Figure 7A) and MUC2-positive (data not shown) goblet-like cells in crypts; lower mRNA levels of multiple Paneth cell markers (Figure 7C, Supplementary Figure 7C, and data not shown); and the presence of a subpopulation of cells with a mixed Paneth/goblet phenotype as shown by co-expression of MUC2 and DEFA-RS (data not shown). *Car1* mRNA abundance was up-regulated in jejunum and duodenum (Figure 7D, Supplementary Figure 7D), whereas that of *Apoa1* was down-regulated in jejunum (Figure 7D); *Apoa1* mRNA was not detected in control or knockout duodenum. These data indicate that deletion of both *Gata6* and *Gata4* produces throughout the small intestine all the changes observed in the *G6del* ileum.

Discussion

We have previously shown that GATA4 specifically regulates jejuno-ileal differences in absorptive enterocyte gene expression and function.^{16, 22} Here, we show that in ileum, where *Gata6* is expressed but *Gata4* is not, conditional deletion of *Gata6* results in a decrease in cellular proliferation in crypts, a decrease in enteroendocrine cell allocation, a conversion of Paneth cells into goblet-like cells at the base of crypts, and an alteration in the expression of specific absorptive enterocyte genes that are distinct from GATA4-specific targets. In jejunum and duodenum, where *Gata6* and *Gata4* are co-expressed, conditional deletion of *Gata6* did not produce this phenotype. Instead, Paneth cells were increased, perhaps as a compensatory response to the loss of Paneth cells in ileum. When both *Gata6* and *Gata4* were conditionally deleted, the proximal intestine exhibited all the changes in proliferation, differentiation and gene expression found in the ileum of single *Gata6* conditional knockout mice. These data indicate that while GATA4, but not GATA6, specifically controls jejunal vs. ileal identities in absorptive enterocyte gene expression,^{16, 21, 22} GATA6 and GATA4 share common functions in regulating crypt cell proliferation and secretory cell differentiation. GATA6 and GATA4 have common and distinct functions in regulating the expression of specific absorptive enterocyte genes. We believe that GATA6 and GATA4 mediate their effects on proliferation and lineage differentiation by acting within stem and/or progenitor cells in crypts, and on absorptive enterocyte gene expression by mechanisms that occur within these cells on villi.

Cellular proliferation in intestinal crypts is necessary for the continuous renewal of the intestinal epithelium. Our data show that GATA6 or GATA4 is necessary for the maintenance of villus height and epithelial cell number, and of crypt cell proliferation throughout the small intestine. Because apoptosis on villi (or in crypts) was not increased, we believe that the observed decrease in villus height and cell number is a direct result of the decrease in crypt cell proliferation in which the rate of epithelial cell renewal is reduced. Although GATA factors have been shown to regulate cellular proliferation in other non-intestinal systems,³⁵⁻³⁹ our data are the first to show a role for this family of transcription factors in cellular proliferation in the small intestine. The decrease in villus height and cell number likely results in a reduction in intestinal surface area, which could adversely affect nutrient absorption, though we did not observe differences in body weight over the 4 weeks of *Gata6* deletion. Taken together, these data indicate that GATA6 or GATA4 is necessary to maintain the normal regeneration of the intestinal epithelium by maintaining its proliferative capacity.

The precise allocation and orderly differentiation of specialized cell types along the crypt-villus axis are essential for establishing the functional landscape of the intestinal epithelium. Here, we also show that GATA6 or GATA4 is necessary for secretory cell differentiation. In *G6G4del* mice, we demonstrate normal goblet cell differentiation and migration onto villi, but a decrease in enteroendocrine and Paneth cells, and a gain of goblet-like cells in crypts. The loss of enteroendocrine cells, indicated by an overall decrease in the number of CHGA-positive cells and *Ngn3* gene expression, suggests a decreased commitment to this lineage. Thus, GATA4 or GATA6 is necessary for enteroendocrine cell commitment in secretory progenitors.

The normal differentiation of goblet cells onto villi, and the loss of Paneth cells and gain of goblet-like cells in crypts where Paneth cells normally reside, suggests a defect in the Paneth cell differentiation program. Paneth cell loss and goblet-like cell accumulation did not occur until at least 2 weeks after the induction of *Gata6* deletion (Supplementary Figure 5), consistent with the slower turnover rate of Paneth cells. The goblet-like cells that accumulate in crypts display a nearly complete spectrum of goblet cell characteristics, but

are different from fully differentiated goblet cells on villi in that they do not express TFF3 (Figure 2C). The goblet-like cells also express genes that promote Paneth cell differentiation and their crypt localization, including SOX9 and EPHB3 (Figure 3A), respectively, which are not normally expressed in mature goblet cells. A small percentage of cells also have mixed goblet-Paneth features (Figure 2C and F). We believe that the goblet-like cells that accumulate in the crypts are committed Paneth cells that, in the absence of GATA6 and GATA4, fail to differentiate further, and by default differentiate to a goblet-like cell-type. Together, these data indicate that GATA4 or GATA6 promote the differentiation of Paneth cells in committed (SOX9) and targeted (EPHB3) Paneth progenitors by preventing their default to a goblet-like cell type.

Wnt signaling is required for crypt maintenance and expression of specific Paneth cell genes.^{4, 5, 27, 40} Although nuclear β -catenin is undetectable in the goblet-like cells in the crypts and the mRNA abundances of Paneth-specific Wnt targets are reduced in the ileum of *Gata6del* mice, other (non Paneth-specific) crypt targets are either unaffected (*c-Myc*, *Lgr5*, *Ephb2*, *Ephb3*) or up-regulated (*Cd44*, *Sox9*) by *Gata6* deletion (Figure 3). We therefore conclude that overall intestinal Wnt signaling is not compromised by *Gata6/Gata4* deletion. The apparent loss of Paneth-specific Wnt signaling is likely due to the loss of fully differentiated Paneth cells, in which expression of the gene encoding FZD5, the Paneth-specific Wnt receptor,²⁷ is reduced (Figure 3B).

Analysis of Notch signaling reveals a shift toward a goblet cell differentiation program, as indicated by an up-regulation of *Spdef*, and down-regulation of both *Ngn3* and *Dll1* (Figure 4A). Over-expression of *Spdef* causes an increase in goblet cells and a decrease in enteroendocrine and Paneth cells, as well as a decrease in crypt cell proliferation,³³ a phenotype that is strikingly similar to that in our models. Conditional deletion of *Spdef* impairs goblet and Paneth cell maturation, and causes an accumulation of secretory progenitors, as indicated by more crypt cells expressing DLL1.³⁴ Deletion of *Dll1* leads to an increase in goblet cells in zebrafish intestine.⁸ It is thought that DLL1 laterally inhibits the secretory program in adjacent cells by activating Notch signaling and the absorptive enterocyte differentiation program. These data suggest that SPDEF promotes enteroendocrine cell allocation and Paneth cell differentiation, and inhibits goblet cell differentiation, possibly by down-stream regulation of *Ngn3* and *Dll1*. Our data are consistent with the hypothesis that GATA6 or GATA4 maintains the balance of secretory cells by repressing *Spdef* gene expression in secretory progenitors. Since *Gata6* mRNA abundance was unaffected by either *Gfi1* or *Spdef* deletion or *Spdef* over-expression (Figure 4C), we conclude that GATA6 or GATA4 act independently of GFI1 and upstream of SPDEF to regulate secretory cell differentiation.

Previously, we showed that conditional deletion of *Gata4* results in a jejunum-to-ileum transformation in absorptive enterocyte gene expression.^{16, 22} These changes occur in the presence of GATA6,¹⁶ indicating that GATA6 cannot replace GATA4 to regulate specific GATA4 gene targets. Here, we show that in the ileum, GATA6 or GATA4 activate and repress specific absorptive enterocyte genes that are different from the GATA4-specific targets (Figure 5). Many of the genes down-regulated in *G6del* ileum encode lipid transporters and apolipoproteins, suggesting that *Gata6* regulates ileal lipid metabolism (Supplementary Figure 4C), though we did not detect alterations in serum cholesterol or triglyceride levels (data not shown). Many of the genes up-regulated by conditional *Gata6* deletion are normally more highly expressed in colon than small intestine. These data suggest that GATA factors regulate intestinal lipid metabolism, and maintain the proximal-distal transcriptome in the small intestine by distinguishing jejunal vs. ileal (GATA4-specific) and intestinal vs. colonic (GATA4 or GATA6) gene expression. These data also

suggest that the expression of specific absorptive enterocyte genes is regulated by mechanisms that involve differential recruitment of specific GATA factors.

Renewal of the intestinal epithelium with precise cell distribution and gene expression patterns is tightly regulated by multiple factors and pathways.⁴¹ In this study, we add to this list of regulators by showing that GATA6 or GATA4 are required for intestinal proliferation, secretory cell differentiation and absorptive enterocyte gene expression. GATA factors are thus critical for intestinal regeneration, and could play a role in damage repair and the adaptive response after loss of functional intestinal surface, or in defects in proliferation, as in intestinal neoplasms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APOA1	apolipoprotein A-I
ASBT	apical sodium-dependent bile acid transporter
ATOH1	atonal homolog 1
BrdU	bromodeoxyuridine
CAR	carbonic anhydrase
CD44	CD44 antigen
CHGA	chromogranin A
c-MYC	myelocytomatosis oncogene
DEFA1	alpha-defensin 1
DEFA4	alpha-defensin 4
DEFA-RS	alpha-defensin related sequence
DLL1	delta-like 1
EM	electron microscopy
EPHB2	eph receptor B2
EPHB3	eph receptor B3
FZD5	frizzled 5
GCG	glucagon

GFI1	growth factor independent 1
HES1	hairy and enhancer of split 1
LGR5	leucine rich repeat containing G protein coupled receptor 5
LYZ	lysozyme
mRNA	messenger RNA
MUC2	mucin 2
NGN3	neurogenin 3
PYY	peptide YY
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
SOX9	SRY-box containing gene 9
SPDEF	SAM pointed domain containing ets transcription factor
TFF3	trefoil factor 3

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Figure 1.

Intestinal *Gata6* deletion results in a reduction in proliferation in the mature ileum. (A) From hematoxylin and eosin stained slides, crypt-villus junctions were established (dotted line) and villus height and crypt depth (brackets), as well as villus and crypt cell number, were determined (left panel) as indicated in Materials and Methods. Villus height and cell number were decreased in *G6del* as compared to *Control* ileum (right panel). (** $P < .01$, *** $P < .001$, as compared to *Control*). (B) Immunostaining for Ki67 (left panel) reveals that the number of positive cells was decreased after *Gata6* deletion (right panel). (*** $P < .001$, as compared to *Control*). (C) Immunohistochemistry for bromodeoxyuridine (BrdU) (left panel)

demonstrates a decrease in the number of positive cells after *Gata6* deletion (*left panel*). (* $P < .05$, as compared to *Control*, n=2 in each group).

Figure 2.

Intestinal *Gata6* deletion results in an increase in goblet-like cells in crypts, and a decrease in Paneth and enteroendocrine cells. (A) Quantitative RT-PCR shows a decrease in enteroendocrine marker transcripts; number of CHGA-positive cells are decreased in *G6del* ileum. (B) Number alcian blue-positive cells is increased in crypts. (C) Immunostaining for MUC2, TFF3 and LYZ shows an increase and MUC2 and decrease in LYZ in crypts; electron microscopy shows an increase in goblet-like cells in crypts; co-immunofluorescence shows co-localization of DEFA-RS and MUC2 in crypts of *G6del* mice. (D) Quantitative RT-PCR of *Muc2* and *Tff3* shows an increase in *Muc2* mRNA abundance. (E) Number of LYZ-positive cells in crypts and *Lyz* mRNA abundance are both

decreased in *G6del* ileum. (F) Electron microscopy shows the presence of cells with mixed goblet/Paneth granules in *G6del* ileum. * $P<.05$, ** $P<.01$, *** $P<.001$, as compared to *Control*.

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Figure 3.

Intestinal *Gata6* deletion results in changes in the expression of crypt Wnt targets in ileum. (A) Immunostaining reveals a decrease in nuclear β -catenin, expression of EPHB3, and an increase in intensity of SOX9 in crypts of *G6del* ileum. (B) Quantitative RT-PCR shows changes in the mRNA abundance of specific proteins involved in intestinal Wnt signaling. (* $P < .05$, ** $P < .01$, as compared to *Control*).

Figure 4.

Intestinal *Gata6* deletion results in alterations in Notch signaling targets. (A) Quantitative RT-PCR shows changes in the mRNA abundance of specific proteins involved in intestinal Notch signaling. (B) Immunostaining reveals an increase in SPDEF in crypts of *G6del* ileum. (C) Quantitative RT-PCR shows no change in the mRNA abundance of *Gata6* in *Gfi1* knockout (*Gfi1*^{-/-}), *Spdef* knockout (*Spdef*KO) and *Spdef* over-expressing (*Spdef*TG) mice. *P<.05, **P<.01, ***P<.001, as compared to *Control*.

Figure 5.

Intestinal *Gata6* deletion results in alterations in the expression of specific absorptive enterocyte genes. (A) Quantitative RT-PCR shows no change in *Asbt*, an increase in *Car1*, and a decrease in *ApoA1* mRNA abundances in *G6del*. ** $P < .01$, *** $P < .001$, as compared to *Control*. (B) Immunostaining shows an increase in CAR1 in absorptive enterocytes of *G6del* ileum.

Figure 6. Intestinal *Gata6* deletion results in an increase in Paneth cells in the jejunum. (A) Quantitative RT-PCR shows increases in *Lyz*, *Defa1* and *Defa4* mRNA abundance after *Gata6* deletion. ** $P < .01$, *** $P < .001$, as compared to *Control*. (B) Immunohistochemistry revealed an increased intensity and a more widespread pattern of LYZ staining in jejunal crypts after *Gata6* deletion. (C) Electron microscopy revealed more Paneth cells and Paneth granules that were more variable in size and more widely dispersed in *G6del* ileum.

Figure 7.

GATA4 is redundant for most GATA6 functions in the mature jejunum. (A) Sections stained for Ki67 and alcian blue revealed (B) a decrease in the number of Ki67-positive cells per crypt in *G6G4del* as compared to *Control* jejunum. (C) Quantitative RT-PCR shows decreases in *Ngn3* and *Lyz* mRNA abundances in *G6G4del* jejunum. (D) Quantitative RT-PCR shows an increase in *Car1* and a decrease in *ApoA1* mRNA abundance in *G6G4del* jejunum. * $P < .05$, ** $P < .01$, *** $P < .001$, as compared to *G6del* jejunum.