GATA TRANSCRIPTION FACTORS AND THE REGULATION OF INTESTINAL DEVELOPMENT, DIFFERENTIATION AND FUNCTION

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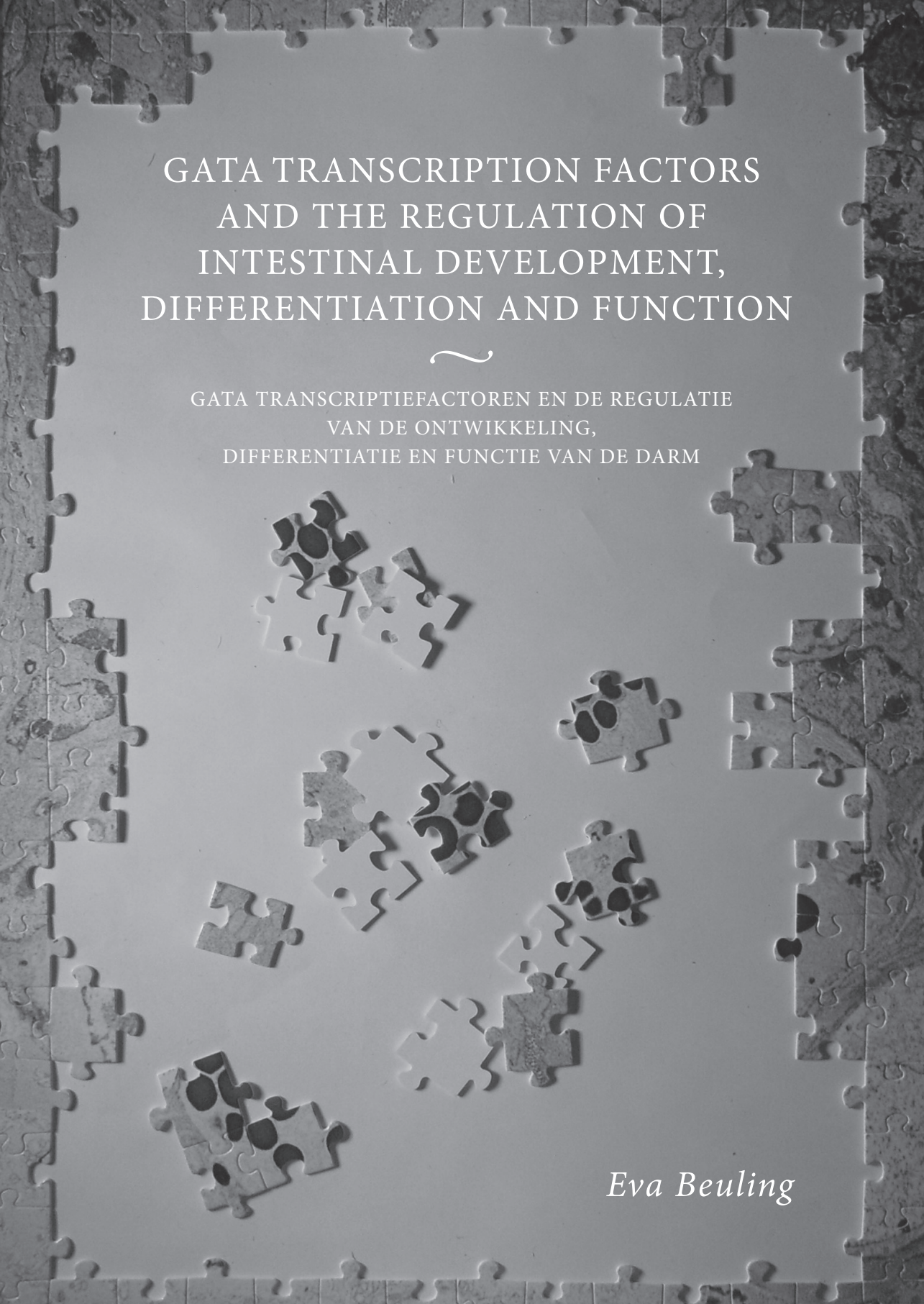
GATA TRANSCRIPTIEFACTOREN EN DE REGULATIE VAN DE ONTWIKKELING, DIFFERENTIATIE EN FUNCTIE VAN DE DARM



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GATA TRANSCRIPTION FACTORS AND THE REGULATION OF INTESTINAL DEVELOPMENT, DIFFERENTIATION AND FUNCTION

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General Introduction
Intestinal development and differentiation

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The mammalian intestine is responsible for the absorption of dietary nutrients and water, and the excretion of waste materials. While selective exchange of compounds takes place between the intestinal lumen and tissue, the intestine also has an important barrier function in denying access to less desirable substances.[1] To facilitate these specified and diverse functions, the lumen of the intestine is lined by a highly differentiated epithelium comprised of specialized absorptive and secretory cells that display a wide-ranging, yet tightly regulated diversity in distribution and gene expression along the cephalocaudal axis. This spatial diversity results in a well-organized series of events taking place in different regions of the intestine leading to an exquisite efficiency in the absorption of all necessary nutrients and water.[2] Disease processes, congenital deviations or inescapable resections, however, easily disrupt these events. Thus, to eventually develop strategies to regenerate lost or deficient intestinal function when gastrointestinal processes go awry, it is essential to completely understand the molecular mechanisms underlying the development and maintenance of the epithelium required for physiological functioning of the intestine.

DEVELOPMENT AND MAINTENANCE OF THE INTESTINAL EPITHELIUM

The intestinal epithelium is a continuous lining on the luminal surface of the small and large intestine and is comprised of a monolayer of specialized columnar cells that are organized in a well-defined fashion supported by the underlying mesenchyme. There are two important components to the structure of the intestinal epithelium: First, the epithelium of the small intestine is organized into folds, that contain fingerlike projections, the so-called crypt-villus units, to maximize the surface area for the exchange of compounds. Cells that are localized to the crypts proliferate and give rise to cells with digestive, absorptive and secretory characteristics that migrate up and form the villus epithelium or reside at the bases of the crypts. In the large intestine, these units consist of only crypts, and no villi, with absorptive and secretory functions contained within them. These units are referred to as the radial (or in the small intestine the crypt-villus) axis of the intestinal epithelium. Second, there are structural characteristics, such as cell type distribution, that differ along the length of the intestine. These morphological differences, in combination with differences in the expression of proteins with specialized functions along the length of the intestine, correlate with differences in functionality in different regions of the intestine, and are referred to as regional or spatial differences along the cephalocaudal axis. This structural organization, with differences in cell distribution and protein expression along the cephalocaudal axis, is established during development and is maintained throughout adulthood.

Intestinal morphogenesis: from primitive gut to differentiated mature intestine

During embryonic development, the mammalian intestinal epithelium is formed from the visceral endoderm through a series of programmed transitions.[3] In early embryonic development, the visceral endoderm, lining the mesoderm and ectoderm, undergoes a series of anterior and posterior invaginations to form the primitive gut tube.[4] The primitive gut, at this stage a layer of cuboidal endodermal cells forming a straight tube, undergoes considerable longitudinal growth and subdivides into three regions along the cephalocaudal axis,[4] the foregut, the midgut and the hindgut, during the so-called patterning phase.[5] The foregut will give rise to the pharynx, esophagus, stomach, and proximal duodenum; the midgut will produce the remainder of the duodenum, small intestine and proximal colon; and the hindgut will develop into the distal colon and rectum.

Once the patterning of the intestinal tract has taken place, longitudinal growth continues and the epithelium of the small intestine remodels through a process of epithelial and mesenchymal reorganization in a proximal to distal progression to form characteristic finger-like projections (villi) and intervillus regions.[6] This epithelial and mesenchymal reorganization coincides with cytodifferentiation of the epithelium along the radial axis, in which the undifferentiated, stratified epithelium is transformed into a highly differentiated columnar epithelium. Fast amplifying progenitor cells segregate to the intervillus regions and give rise to the absorptive and secretory cells.[7] Differentiated cells migrate upwards or basally and begin to express proteins that are critical for the digestive, absorptive, and secretory functions of the intestine after birth.[6]

Postnatally in rodents and in the second trimester during pregnancy in humans, the proliferative intervillus regions of the fetal small intestine develop into the crypts of Lieberkühn and the crypt-villus structure fully develops.[6] The crypts of Lieberkühn contain a crypt base and a distinct proliferating compartment with transit amplifying cells that give rise to absorptive enterocytes, goblet cells and enteroendocrine cells that differentiate while migrating upwards, and Paneth cells that differentiate while migrating down to the crypt base.[8] The development of the colonic epithelium is very similar to that of the small intestine. The colonic epithelium also starts out as primitive stratified epithelium that is converted to a villus architecture with developing crypts, but then it undergoes another important cytodifferentiative transition in which villi disappear and the adult type crypt epithelium that consists of colonocytes, goblet cells and enteroendocrine cells, but no Paneth cells, expands.[6]

After development, when the intestine is fully formed, the crypt-villus structure in the small intestine and the crypt epithelium in the colon are maintained through a continuous renewal process, in which stem cells located near the bases of the crypts produce transit amplifying cells that continuously give rise to the differentiated intestinal epithelial cells.[8] In the mature small intestine, the absorptive enterocytes are the majority cell type comprising more than 80% of all epithelial cells and expressing transporters and digestive enzymes to fulfill their absorptive function. Goblet cells, which constitute 4-12% of all epithelial cells, are localized from the mid-crypt to the villus tip and secrete mucus to protect the lumen of the small intestine against mechanical and chemical damage. Enteroendocrine cells are scattered as individual cells throughout the epithelium forming approximately 1% of all crypt-villus cells; they secrete hormones that regulate gastrointestinal processes, such as gastric secrection and emptying, pancreatic secretion, and intestinal motility. Paneth cells, which represent 3-8% of all crypt cells are located at the base of the crypts of Lieberkühn and secrete antimicrobial molecules that participate in the innate immune defense system. The intestinal epithelium is the organ with the highest regenerative rate in vertebrates. Upon reaching the tips of the villi, absorptive enterocytes, goblet cells and enteroendocrine cells undergo apoptosis, exfoliate and are shed into the intestinal lumen. The cell migration rate is approximately 3-4 days.[9] Paneth cells reside at the base of crypts and turn over at a slower rate of 3-6 weeks.[9-13] In the colon, colonocytes that mainly function to absorb water and electrolytes are the predominant cell type in the epithelium, with a turnover rate of 5-8 days.[9, 14] The colon also contains goblet cells and enteroendocrine cells, like the small intestine, but no Paneth cells. Goblet cells and enteroendocrine cells in the colon turnover every 5-8 days, at a similar rate as the colonocytes. The ascending colon has an additional cell type called deep crypt secretory cells which migrate inwards and their turnover time is about 14-21 days.[9]

Regional differences in intestinal functionality

The patterning along the length of the intestinal epithelium that takes place during development results in a mature intestine that is divided into functionally different regions in which a well-organized series of events take place that lead to an exquisite efficiency in the absorption of all

necessary nutrients and water, the excretion of waste materials, and repulsion of less desirable substances. The small intestine is divided into three regions; duodenum, jejunum, and ileum. The duodenum begins immediately after the gastric pylorus and extends to the ligament of Treitz.[15] The main function of the duodenum is the initiation of the process of digestion and the luminal absorption of iron, calcium, and water soluble vitamins. In addition, hormones secreted by the duodenum regulate the rate of stomach emptying.[16] The jejunum starts at the ligament of Treitz and is the middle of the three small intestinal regions. Its main function is the absorption of nutrients from the luminal contents. The final section of the small intestine, the ileum, is separated from the cecum by the ileocecal valve. The main function of the distal ileum is the absorption of residual nutrients not taken up by the jejunum, and the carrier mediated transport of bile acids[17] and vitamin B12.[18] The cecum separates the small intestine from the colon, and collects waste from the small intestine. The colon is mainly responsible for concentration of fecal effluent through water and electrolyte absorption. Furthermore, the colon facilitates storage and controlled evacuation of fecal material, and its bacteria are required for the digestion and absorption of otherwise undigested food. Additional roles for the gut flora in intestinal function are under intense study.[19]

Gene expression during intestinal development and in the mature intestine

To facilitate its highly specialized functions, the intestine requires the synthesis of proteins such as digestive enzymes, transporters, cytoplasmic carriers, mucus, hormones, and antimicrobial peptides providing the differentiated cells with specific functions. Differences in cell distribution and gene expression within differentiated cells along the length of the intestine are established during development, change to correlate with the functional needs of the intestine in different phases of development, and reach a stable state that is maintained throughout adulthood.

In the first postnatal period, mammals are dependent on milk for their nutrition. During postnatal development there is a transition from dependence on nutrients from milk to dependence on nutrients from a diet of solid food. This conversion is inherent to mammals, is referred to as the weaning transition, and is "hard-wired" and not induced by food.[20] To adequately digest and absorb nutrients from different sources, the expression patterns of proteins required for some of these functions change during these transitions. This is exemplified by the expression patterns of lactase-phlorizin hydrolase (LPH), a member of the β-galactosidase family of enzymes that is crucial for the digestion of lactose in milk, and sucrase-isomaltase (SI), an enzyme that is responsible for the digestion of α-disaccharides found in solid foods. In rodents, LPH is expressed in absorptive enterocytes,[21-25] is first detected during cytodifferentiation of the intestinal epithelium,[24, 26] and is highly expressed along the total length of the small intestine at birth and during suckling.[22, 25] During the weaning transition, a marked reduction of LPH expression takes place. At the time of weaning, *Lph* mRNA is no longer detectable in the duodenum or terminal ileum, and LPH expression is reduced in the mid small intestine.[22, 25] In contrast, SI is not expressed until after birth,

and its expression increases during the weaning transition, reaching its highest level throughout the small intestine in adulthood.[22, 25]

In the mature intestinal epithelium, the distribution of cell types and the expression patterns of proteins with specific functions along the cephalocaudal axis is maintained and results in regional differences in functionality. For example, goblet cells[27] and Paneth cells[10] are more numerous in distal small intestine. Paneth cells express a different repertoire of antimicrobial molecules along the length of the small intestine. Whereas alpha-defensin 1(DEFA1) is the main defensin in the proximal small intestine, alfa-defensin 4 (DEFA4) is the most highly expressed in the distal small intestine.[28] Enteroendocrine subpopulations display a functional diversity characterized by the regional segregation of hormones that activate (e.g. cholecystokinin, CCK) or repress (e.g. peptide YY, PYY) gastrointestinal processes.[29] CCK is synthesized mostly in the proximal small intestine and its secretion stimulates gastric emptying, pancreatic secretion, and increases intestinal motility, whereas PYY is secreted by endocrine cells located in the distal small intestine, leading to inhibition of digestive processes and intestinal motility. Absorptive enterocyte genes that encode proteins responsible for the terminal digestion and absorption of nutrients can be separated into at least four groups based on patterns of expression: (a) genes expressed mainly in proximal intestine such as duodenal ferroportin, (b) genes that are expressed throughout the small intestine, as exemplified by SI[30] and intestinal fatty acid binding protein (FABP2)[31], (c) genes that are expressed in mid small intestine, as shown by LPH[23] and liver fatty acid binding protein (FABP1)[32], and (d) genes that are expressed in distal small intestine, such as the apical sodium-dependent bile acid transporter (ASBT) responsible for the absorption of bile acids[33] and ileal lipid binding protein (ILBP).[34] These and other regional expression patterns regulate the absorption of nutrients in proximal and mid small intestine. In contrast, substances such as bile acids and cobalamin that may be required for function along the length of the small intestine are not absorbed until they have reached the distal ileum.

In the large intestine, the cell distribution and expression patterns of proteins facilitate the colonic absorption of large quantities of water. The fundamental feature that enables efficient water transport is the ability of the colonocytes to generate a large osmotic gradient between the lumen and the intercellular space, mediated by energy-dependent Na+/K+-ATPase pumps. This process of colonic Na^+ and water absorption is mediated by parallel apical membrane Na^+/H^+ and Cl^-/HCO_3^- exchange, and facilitated by aquaporin.[35] NHE2 and NHE3 (also known as solute carrier family 9 (sodium/hydrogen exchanger), member 2 and 3, SLC9A2 and SLC9A3), are responsible for Na^+ and H^+ exchange. Their spatial distributions are different, as NHE2 is expressed in both surface and crypt cells, while NHE3 is present only in surface cells.[36] Also exclusively in these surface cells are the colonic Cl^-/HCO_3^- exchangers AE1 and AE2 (also known as solute carrier family 4 (anion exchanger), member 1 and 2, SLC4A1 and SLC4A2).[37] The primary cellular source of H^+ and HCO_3^- ions needed to supply these exchangers is the enzyme carbonic anhydrase (CAR). This enzyme catalyzes the hydration of carbon dioxide, creating H^+ and HCO_3^- simultaneously

and is highly expressed in the apical membrane (CAR4) and cytoplasm (CAR1 and CAR2) of colonocytes.[38, 39]

MOLECULAR MECHANISMS UNDERLYING THE DEVELOPMENT AND DIFFERENTIATION OF THE INTESTINAL EPITHELIUM

The formation of the intestinal epithelium during development, and its maintenance throughout adulthood, is controlled by evolutionarily conserved mechanisms. Before the gut tube undergoes patterning and organogenesis, certain transcription factors are expressed in an asymmetrical manner marking presumptive territories for the different gut regions.[40] These factors are also involved in instructing cells as to their positions on the proximal-to-distal axis and determining their cell fate. Some of these transcription factors, such as the homeobox (*Hox*) genes, are predominantly expressed in the mesoderm, where they control the regional secretion of molecules that in turn signal to the adjacent endoderm, whereas other transcription factors localized to the endoderm control the secretion of signaling peptides, such as Sonic Hedgehog, which in turn activate *Hox* genes in the mesenchyme. Crosstalk between different factors in the mesenchyme and epithelium at different time points in development is therefore thought to be critical for regionalization and differentiation of the intestinal epithelium. A number of the factors that are involved in early organogenesis, such as the forkhead-related factors (*Fox* genes) and GATA factors, are also required for other steps in the development and maintenance of the intestinal structure. Signaling pathways, such as the hedgehog pathways and those mediated by members of the TGFβ superfamily of growth factors, including transforming growth factor β (TGFβ) and the bone morphogenetic proteins (BMP), act at different times and in different locations to regulate specific phases of the development and maintenance of the intestinal epithelium.[6]

Regulators of early embryonic patterning

The homeotic genes encode homeobox containing transcription factors. The role of these transcription factors is well characterized in *Drosophila,* where they are expressed in a precise cephalo-to-caudal order and control segmentation and pattern formation. Homologs of this family of transcription factors in vertebrates, the so-called *Hox* genes, are also expressed in a regional manner and are important for determining morphologic boundaries of different organs. Disruption of the expression of specific *Hox* genes results in organ-specific gastrointestinal defects. Disruption of *hoxc-4* displays obstruction of the esophagus due to abnormal muscle development and altered epithelial cell proliferation.[41] Ectopic anterior localization of *Hox3.1* (now *Hoxc-8*) expression in the stomach results in distorted gastric epithelial development,[42] whereas the loss *hoxa-5* in the mesenchyme alters gastric epithelial cell.[43] Disruption of the *hoxd-12* and *hoxd-13* genes in mice leads to defects in anal musculature formation.[44] Together these findings indicate that *Hox* genes are essential for early morphogenesis of certain gut regions. Whereas almost all

Hox genes analyzed are localized to mesodermal tissue, they are likely affecting endodermal development through interactions between mesenchyme and epithelium. Although the disruption of a number of *Hox* genes leads to malformations of specific regions of the gastrointestinal tract, these mutations do not lead to an overall cephalocaudal transformation of the gut,[45] suggesting that additional factors are required for the complete patterning of the intestine.

Regulators of intestinal patterning

In addition to the *Hox* gene cluster, the *ProtoHox* cluster gives rise to the *ParaHox* cluster.[46] The *ParaHox* gene cluster, that includes pancreatic-duodenum-homeobox 1 (*Pdx1*) and caudal type homeobox (*Cdx*) genes, plays critical roles in cephalocaudal endoderm patterning by controlling *Hox* gene expression as shown in Drosophila, Caenorhabditis elegans and Xenopus. PDX1 is first expressed when the foregut commits to a pancreatic fate and is essential for pancreas formation,[47] whereas the *Cdx* genes are important for intestinal epithelial development. CDX1 is first expressed in the ectoderm and mesoderm of the primitive streak. The expression of CDX1 is lost temporarily, returns throughout the intestinal epithelium later in development, and becomes restricted to the proliferative crypt compartment during postnatal differentiation and in the mature intestine.[48] CDX2 is expressed much earlier than CDX1 and its expression becomes restricted to the endoderm of the primitive gut.[49] Homozygous mutations in *Cdx1* do not result in gross intestinal defects during development,[50] while germ line *Cdx2* null mice die before the endoderm develops.[51] *Cdx2* heterozygous mice display polyps in the colon containing squamous metaplasia.[51, 52] Furthermore, it has been shown that ectopic expression of CDX2 in the stomach leads to intestinal metaplasia.[53] Conditional deletion of *Cdx2* specifically in early endoderm results in a transformation of the caudal intestinal epithelium to an esophageal type of epithelium, with no colon formation. This demonstrates that CDX2 is crucial for determining the organ boundaries on the cephalocaudal axis of the intestine by controlling the onset and/or maintenance of the expression of intestinal transcription factors that regulate the intestinal transcriptome, such as CDX1 and hepatocyte nuclear factor 1α (HNF1α).[45]

Regulators of the development and maintenance of a functional crypt-villus structure

After early patterning has taken place, the intestinal epithelium transitions from an undifferentiated, stratified epithelium to a highly differentiated columnar epithelium, and the formation of crypt-villus units starts. A model is developing on how multiple molecular signaling pathways, such as canonical Wnt, Notch and Hedgehog cooperatively establish and maintain a functional crypt-villus structure by tightly regulating cellular proliferation, differentiation, migration and cell death. These pathways are based on ligands binding to their receptors on the membrane of the signal receiving cells. The Wnt and Hedgehog ligands are secreted and bind to their receptors in a paracrine or endocrine manner, while the Notch ligands are expressed in cell membranes and interact with the transmembrane receptors of adjacent cells. Ligand-receptor binding initiates a

signaling cascade inside the cells resulting in transcriptional regulation of target genes in the nuclei. The localization, abundance and timing of active signaling, as well as the interaction between the pathways determine the effects on the signal-receiving cells.

Central to the canonical Wnt signaling cascade is the tight regulation of degradation versus stabilization of cytoplasmic β-catenin. In the absence of Wnt signaling, a degradation complex, consisting of axin, glycogen synthase kinase 3α/β (GSK3α/β), casein kinase 1-γ (CK1γ) and adenomatosis polyposis coli (APC), phosphorylates cytoplasmic β-catenin, leading to its ubiquitination and degradation in proteosomes. Binding of Wnt glycoproteins to their trans-membrane coreceptors, the frizzled proteins (FZD) and the LDL receptor-related proteins 5 and 6 (LRP5/6) initiates a signaling cascade in which the inhibition of the degradation complex leads to the stabilization and nuclear translocation of β-catenin. In the nucleus, β-catenin interacts with the TCF/LEF family of transcription factors to regulate the expression of Wnt-responsive genes.[7] Inactivation of Wnt signaling by expressing the Wnt protein inhibitor dickkopf 1 (DKK1) leads to reduced proliferation with crypt loss and the absence of secretory lineages in adult small intestine,[54] whereas deletion of TCF4 leads to the loss of proliferative compartments and enteroendocrine cells during embryonic gut development.[55] Constitutive activation of Wnt signaling through mutations in APC[56-59] or other mutations that lead to the stabilization of β-catenin[60] results in increased proliferation and adenomatous polyp formation in the mouse intestine. Together these studies reveal that Wnt signaling generally promotes intestinal precursor cell proliferation and crypt formation, and is required for secretory cell lineage commitment.

Notch signaling is based on cell-to-cell contact between cells that express Notch receptors and adjacent cells that express Notch ligands. Notch receptors (Notch1-4 in mammals) are transmembrane proteins that consist of an extracellular and an intracellular domain. Binding of Notch ligands (delta-like1through 3 and jagged 1 and 2 in mammals, also transmembrane proteins) to the extracellular domain of Notch receptors leads to the cleavage of the intracellular domain of the receptor by γ-secretase.[61] The intracellular cytoplasmic domain (ICD) translocates to the nucleus where it interacts with the transcription factor recombination signal binding protein for immunoglobulin kappa J region (RBPJ) leading to the recruitment of transcriptional coactivators and expression of Notch-responsive genes such as hairy and enhancer of split (*Hes*) 1 and 5.[62, 63] Blockage of Notch signaling in the intestinal epithelium by inhibition of γ-secretase or by deletion of the gene encoding RBPJ leads to a halt in epithelial cell division and a marked increase in the number of goblet cells at the expense of the absorptive cell lineage.[64] In contrast, activation of Notch signaling by transgenic expression of the Notch intracellular domain results in an expansion of immature progenitor cells and a block of differentiation of secretory cells.[65] Together these studies demonstrate that Notch signaling is required for the maintenance of the undifferentiated state of the crypt progenitors and is essential for absorptive cell commitment.

The hedgehog (HH) signaling pathway is another morphogenetic pathway that regulates the development and maintenance of the gastrointestinal epithelium. Sonic, Indian, and desert hedgehog (SHH, IHH and DHH) are the three HH proteins that have been identified in most

vertebrates. HHs bind to the patched homolog (PTCH) receptor. In the absence of ligand, PTCH inhibits smoothened (SMO), a downstream protein in the pathway. When HH binds PTCH, this inhibitory interaction is relieved and SMO signaling is activated. SMO signaling leads to the activation of the GLI-Kruppel family transcription factors GLI1, GLI2 and GLI3. GLI transcription factors exist both in a full length activator and a truncated repressor form generated by proteolytic processing. SMO signaling blocks the constitutive proteolytic processing of GLI transcription factors, which leads to the stabilization and nuclear translocation of the full length version of the GLIs and transcriptional activation of HH-responsive genes.[66] Inhibition of HH signaling by transgenic expression of the pan-hedgehog inhibitor HHIP (hedgehog interacting protein) leads to increased proliferation and block of enterocyte differentiation.[67] Aberrant induction of HH signaling, by mutating the PTCH receptor, results in the depletion of the precursor cell compartment and premature development of the enterocyte lineage.[68] Together these studies show that HH signaling reduces proliferation and promotes enterocyte cell commitment.

REGULATORS OF INTESTINAL GENE EXPRESSION ALONG THE CEPHALOCAUDAL AXIS OF THE MATURE INTESTINE

As described above, CDX transcription factors are expressed in the developing intestine and play important roles in embryonic patterning. In the mature intestine, CDX1 expression increases along the cephalocaudal axis with the highest levels of expression in the distal colon.[69] CDX1 is restricted to intestinal crypts.[48] CDX2 also increases progressively from duodenum to proximal colon, but is equally expressed in crypt and villus cells.[69] Promoter studies of many intestinal genes that encode proteins involved in terminal digestion and absorption of nutrients have revealed binding sites for the CDX and other transcription factors such as HNF1 and GATA, and subsequent in vitro and cell culture assays have implicated these transcription factors as regulators of these intestinal genes.[70-87] In a number of intestinal genes the binding sites for CDX have been shown to be in close proximity to binding sites for HNF1 and GATA transcription factors, and co-regulation and interaction between the transcription factors have been shown to be required for optimal expression of intestinal genes in vitro.[70, 77, 79, 88]

HNF1 transcription factors

The HNF1 family of transcription factors was first discovered in the liver, where they regulate the expression of hepatic genes.[89] The members of the HNF1 family contain an atypical DNA-binding homeodomain that is a variant on homeodomains found in other homeoproteins, like the *Hox* genes. They bind DNA as dimers to the consensus sequence, GTTAATNATTAAC.[89-91] Thus far, two members of the HNF1 family, HNF1α and HNF1β, have been described, which are both expressed in liver, kidney, pancreatic islets, stomach, and small and large intestine. [90, 92]

Although both HNF1α and HNF1β, bind to HNF1 binding sites in intestinal gene promoters, only HNF1α demonstrates activation of these promoters in transient co-transfection assays,[76, 77, 82, 87] implicating HNF1α as the principle HNF1 factor for intestinal gene regulation. In mice, HNF1α is expressed throughout the small and large intestine, with the greatest abundance in jejunum and ileum. On the radial axis, HNF1α is expressed at high levels in crypt cells and its expression reduces gradually toward the villus tips.[93] HNF1α has been shown to activate the promoter of several genes expressed in the intestine, including phosphoenolpyruvate carboxykinase,[94] aminopeptidase N,[95] guanylin,[76] aldolase B,[96] SI,[87] and LPH.[77, 79, 82]

Mice homozygous for the *Hnf1α* null allele survive, but demonstrate decreased growth, abnormal liver function, and sterility.[97, 98] Like humans with heterozygous mutations in the gene encoding HNF1α,[99-101] these mice develop diabetes.[102] The expression of several hepatic genes, such as albumin, α1-antitrypsin, phenylalanine hydroxylase and FABP1, is reduced or abolished in *Hnf1α* null mice.[103, 104] Furthermore, in the small intestine of *Hnf1α* null mice, the expression of ASBT,[105] calbindinD_{9K},[86] LPH and FABP1[106] are reduced, demonstrating that HNF1α is required for the expression of these intestinal genes.

GATA transcription factors

The GATA family of transcription factors exhibits critical and diverse functions in cellular proliferation, differentiation and gene regulation in multiple organs.[107] They are defined by two evolutionarily conserved zinc fingers that mediate binding to the consensus DNA sequence (A/T) GATA(A/G) and facilitate interaction with other transcription factors and cofactors enabling them to regulate gene transcription. The GATA family is generally categorized into two classes based on expression patterns. GATA1, GATA2, and GATA3 are expressed in developing blood cells and are critical for hematopoiesis,[108] whereas GATA4, GATA5, and GATA6 are expressed together only in cardiac tissue and small intestine, but individually, or in overlapping patterns in stomach, colon, liver, lungs, spleen, ovary, testis, and bladder.[109-113]

To determine the function of GATA4, GATA5 and GATA6, germ line knockout mice were generated. Mice homozygous for the *Gata4* null allele die by embryonic day 9.5 (E9.5) and lack both a primitive heart tube and foregut.[114, 115] *Gata5* knockout mice survive and reproduce despite pronounced genitourinary abnormalities in females,[116] but show no gross abnormalities in intestinal structure (intestinal gene expression was not reported). *Gata6* null mice die before gastrulation.[117, 118]

GATA4, GATA5 and GATA6 are expressed in the intestinal epithelium during development and in adulthood, and have been shown to bind and activate the promoters of intestinal genes in vitro, including *Lph*,[74, 77, 85, 119] *Si*,[70, 77] *Fabp1*,[71] *Fabp2*,[75] and adenosine deaminase (*Ada*).[72] The GATA factors physically and functionally interact with HNF1α to synergistically activate the *Lph* promoter,[77, 88] but only GATA4 and GATA6 are capable of activating this promoter in the absence of HNF1α. Binding assays with extracts from mature mouse intestinal epithelial cells showed that

GATA4 is the principal GATA factor binding to the *Lph* promoter,[84] suggesting GATA4 is the important GATA factor in gene regulation in the small intestine.

Overcoming the embryonic lethality of germline *Gata4* null mice, a conditional, inducible loss-of-function model to inactivate GATA4 was established to determine the function of GATA4 in the mature small intestine.[120] In this model the activation domains of *Gata4* were deleted specifically in the mature small intestine upon tamoxifen treatment. In the jejunum of these mice it was found that (a) specific genes that are normally expressed in jejunum but not in ileum, including *Lph* and *Fabp1*, were down-regulated, (b) specific genes normally expressed equally in the jejunum and ileum, including *Si* and *Fabp2*, were unchanged, and (c) genes normally absent in jejunum but expressed in the ileum, including *Asbt* and *Ilbp*, were up-regulated. These findings demonstrate that GATA4 plays a role in the specification of jejunal-ileal identities in absorptive enterocyte gene expression in the adult mouse small intestine.[120] Furthermore, it was shown that inactivation of GATA4 by deletion of its activation domains results in (a) a significant increase in the number of goblet cells on villi, (b) a significant increase in the mRNA for atonal homolog 1 (ATOH1), a mediator of the secretory cell fate that is also expressed in secretory cells,[121] (c) a modest increase in the mRNA for the principal goblet cell marker, mucin 2 (MUC2), and (d) a redistribution in the mRNAs associated with specific enteroendocrine subpopulations, all towards an ileal phenotype. These studies demonstrate a function for the evolutionarily conserved GATA family in the small intestine in vivo, and specifically show that GATA4 plays a fundamental role in the maintenance of jejunal-ileal identities in the adult mouse small intestine through processes involving gene activation, gene repression, and possibly, cell fate allocation.[120, 121]

RATIONALE AND SPECIFIC AIMS

The preceding introduction outlines the molecular mechanisms that are known to be involved in the development, differentiation and maintenance of the intestinal epithelium required for its physiological functioning and describes how research on the regulation of intestine-specific terminal differentiation markers has led to the current knowledge about the involvement of CDX, HNF1 and GATA transcription factors in these molecular mechanisms. Although much is investigated and described about these molecular processes, to eventually develop strategies to regenerate lost or deficient intestinal function it is essential to completely understand these molecular mechanisms. The overall aim of this dissertation is to investigate the fundamental roles of GATA factors and their co-regulators in the underlying mechanisms that regulate proliferation, differentiation, cell commitment and gene expression on the cephalocaudal axis in the development and maintenance of the intestinal epithelium. The following paragraphs describe the unexplored components of the roles of GATA factors and their co-regulators that have led to the specific aims of this dissertation.

Rationale and specific aim 1

LPH, FABP1, and SI are well established markers for the transitions that occur during intestinal development.[22, 24, 25, 122-127] In rodents, LPH and FABP1 are first detected at the beginning of cytodifferentation, continue to be expressed at high levels during the suckling period, and decline during the weaning transition. In contrast, SI is undetectable before weaning and increases to adult levels during the weaning transition. Thus, LPH and FABP1 are markers of cytodifferentiation during fetal development, whereas LPH, FABP1, and SI are indicators of the well-orchestrated patterns of absorptive enterocyte gene expression that occur during postnatal development. Although GATA4 and HNF1α are each indispensible for the expression of *Lph* and *Fabp1* in the adult jejunum,[106, 120] it is not known if GATA4 and HNF1α are also required for the initial expression and/or the regulation of the expression of these genes during the different phases of intestinal development.

Specific aim 1 of this dissertation is to define the requirement of GATA4 and HNF1α for intestinal differentiation in the developing mouse small intestine, specifically focusing on cytodifferentiation and the weaning transition using the expression of the markers *Lph, Fabp1,* and *Si* as readout for intestinal differentiation in an inducible, intestine-specific *Gata4* activation domain-deletion and a germline *Hnf1α* knockout mouse model.

Rationale and specific aim 2

Friend of GATA (FOG) is an evolutionarily conserved, multi-zinc finger cofactor family whose members physically associate with the N-terminal zinc finger of GATA factors, and mediate GATA function in a broad array of tissues and cell types by repressing the expression of specific GATA target genes.[116, 128, 129] Two members of the FOG family, FOG1 and FOG2, have been discovered in vertebrates, and are each expressed in independent and overlapping patterns in multiple tissues and cell types. *Fog1*$^{-/-}$ and *Gata1*$^{-/-}$ mice reveal common embryonic lethal phenotypes characterized by a failure during hematopoiesis.[130, 131] Using a split two-hybrid screen, a GATA1 mutant (GATA1ki) with a valine-to-glycine substitution at position 205 in the N-terminal zinc finger was identified that has attenuated binding affinity for FOG cofactors, but normal DNA binding function.[132] *Gata1*$^{ki/ki}$ mice that express GATA1ki in place of GATA1 also die from a hematopoietic phenotype,[132] similar to that in *Gata1*$^{-/-}$[130] or *Fog1*$^{-/-}$[131] mice, indicating that the GATA1-FOG1 interaction confers most of the GATA1 and FOG1 function on hematopoiesis. *Fog2*$^{-/-}$ and *Gata4*$^{-/-}$ mice, as well as *Gata4*$^{ki/ki}$ mice, which express a GATA4 mutant with an analogous valine-to-glycine substitution, but at position 217 in GATA4, all reveal common embryonic lethal phenotypes characterized by a failure during cardiogenesis indicating a critical requirement for GATA4-FOG2 interactions. FOG1 also plays a role in cardiogenesis,[133] which presumably reflects cooperation with the GATA4, 5, 6 subfamily. The *Gata4*^{ki} model has also revealed that GATA4-FOG interaction is important in gonadal differentiation[134-136] and gastric epithelial development.[137] However, the role of FOG cofactors in mediating the function of GATA factors in the mature small intestine has not yet been investigated.

Specific aim 2 of this dissertation is to investigate the involvement of FOG cofactors in mediating the function of GATA4 in the mature small intestine by determining the alterations in expression of known GATA4 target genes in an inducible, intestine-specific *Gata4* knock-in model in mice, in which wild-type GATA4 is specifically inactivated in the mature small intestine, but a GATA4 mutant that does not bind FOG cofactors (GATA4ki) continues to be expressed.

Rationale and specific aim 3

Conditional, inducible deletion of the activation domains encoded by *Gata4* (i.e. synthesis of GATA4Δex2)[120] results in a transformation in the expression of specific absorptive enterocyte genes in the jejunum to an ileal pattern. However, it is unknown if this transformation in gene expression in the jejunum translates into a transformation to ileal function as well. One ileal-specific function is the absorption of bile acids. Bile acids are synthesized from cholesterol in the liver, stored in the gall bladder, and released into the small intestine where they form mixed micelles to solubilize biliary and dietary lipids for absorption. After fulfilling their function in the small intestine, bile acids are reabsorbed in the distal ileum via a sodium-dependent active transport process mediated by ASBT, a 48 kD protein that is localized to the brush border membrane of ileal absorptive enterocytes.[33] Bile acids are then returned to the liver via the portal circulation. Although small amounts of bile acids are absorbed by passive diffusion throughout the small intestine and colon, loss-of-function mutations in the *Asbt* gene in humans,[138, 139] targeted deletion of the *Asbt* gene in mice,[140, 141] and ileal resection in animals and humans,[33, 142] are all associated with intestinal bile acid malabsorption and a decreased enterohepatic cycling of bile acids, demonstrating that active transport by ASBT in the distal ileum is the principal mechanism for bile acid absorption in mammals. One specific change after conditional, inducible deletion of the activation domains encoded by *Gata4* is the induction in the jejunum of the expression of ASBT. It is unknown if this induction of ASBT in the jejunum leads to bile acid absorption in this region of the small intestine.

Specific aim 3 of this dissertation is to test the hypothesis that the induction of ASBT in the proximal small intestine following the reduction of GATA4 activity leads to bile acid absorption that is sufficient to correct bile acid malabsorption associated with ileocecal resection, using two recombinant *Gata4* models in which *Asbt* expression is induced to different levels.

Rationale and specific aim 4

GATA4 and GATA6 are both expressed in the adult mouse small intestine[120] and are capable of trans-activating the same subset of intestinal target genes in cell culture over-expression experiments,[71, 74, 77, 143] implicating redundant functions in the intestine. *Gata4* mRNA is expressed at high levels throughout the small intestine, with the exception of the distal ileum, where it is greatly reduced.[72, 84, 120, 144] *Gata6* mRNA is expressed evenly throughout the small intestine.[72, 144] Immunostaining experiments indicate that GATA4 and GATA6 proteins are co-expressed throughout the crypt epithelium,[120] and in absorptive enterocytes on villi.[70, 71, 120, 145, 146] Functional redundancy

between the GATA4 and GATA6 is well documented in cardiac development. GATA4 and GATA6 are co-localized in developing and postnatal myocardium and similarly trans-activate several cardiac promoters.[116] Using an adenovirus-mediated mRNA knock-down strategy in postnatal cardiomyocytes, specific genes, including α- and β-myosin heavy-chain (α- and β-MHC), were preferentially regulated by GATA4,[147] indicating a specific function, whereas expression of other cardiac genes was significantly down-regulated equally by *Gata4* or *Gata6* inactivation, supporting a cooperative function. GATA4 and GATA6 heterodimerize in vitro,[143] and synergistically activate the natriuretic peptide precursor type A and B genes in cardiomyocytes,[147] further supporting a cooperative function. Mice that are heterozygous for *Gata4* or *Gata6* null mutations are viable and do not show any obvious cardiovascular phenotype, but double *Gata4*/*Gata6* heterozygous mice die at E13.5 with a spectrum of cardiovascular defects[148] and loss of both *Gata4* and *Gata6* blocks cardiac myocyte differentiation and results in acardia.[149] Together, these data indicate that GATA4 and GATA6 have specific, overlapping, and co-regulatory functions during cardiac development. However, their roles in the small intestine are less well understood. Although GATA4 and GATA6 are co-expressed in the intestinal epithelium, GATA4 alone is responsible for jejuno-ileal distinctions in absorptive enterocyte gene expression.[120] The role of GATA6 in the mature small intestine is currently unknown.

Specific aim 4 of this dissertation is to determine the functions of GATA6 and the redundant functions of GATA6 and GATA4 in the mature small intestine by investigating altered phenotypes in mouse models in which *Gata6* or both *Gata6* and *Gata4* are inducibly deleted specifically in the mature intestine.

Rationale and specific aim 5

GATA6 is expressed in the mature mouse[72, 150] and human[151] colonic epithelium, and has been shown to regulate the expression of colonic genes in vitro, including nonphagocytic NADPH oxidase 1 (*Nox1*)[152], and 15-lipoxygenase (LOX)-1 (15-LOX-1).[153] Furthermore, GATA6 has been implicated to play a role in the development of colon cancer in humans.[151, 153] However, the exact function of GATA6 in the mature colon is currently unknown.

Specific aim 5 of this dissertation was to define the function of GATA6 in the mature colon by inducibly deleting *Gata6* specifically in mouse intestine and characterizing the altered phenotype of the morphological structure and the expression of genes in the mature colon.

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Chapter 2

GATA4 and HNF1α are partially required for the expression of specific intestinal genes during development

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ABSTRACT

The terminal differentiation phases of intestinal development in mice occur during cytodifferentiation and the weaning transition. Lactase-phlorizin hydrolase (LPH), liver fatty acid binding protein (FABP1) and sucrase-isomaltase (SI) are well characterized markers of these transitions. We have previously shown in mature jejunum using gene inactivation models that *Gata4* and *Hnf1α* are each indispensable for *Lph* and *Fabp1* gene expression, but are both dispensable for *Si* gene expression. In the present study, we used these models to test the hypothesis that GATA4 and HNF1α regulate *Lph*, *Fabp1* and *Si* gene expression during development, specifically focusing on cytodifferentiation and the weaning transition. Inactivation of *Gata4* had no effect on *Lph* gene expression during either cytodifferentiation or suckling, whereas inactivation of *Hnf1α* resulted in a 50% reduction in *Lph* gene expression during these same time intervals. Inactivation of *Gata4* or *Hnf1α* had a partial effect (~50% reduction) on *Fabp1* gene expression during cytodifferentiation and suckling, but no effect on *Si* gene expression at any time during development. Throughout the suckling period, we found a surprising and dramatic reduction in GATA4 and HNF1α protein in the nuclei of absorptive enterocytes of the jejunum despite high levels of mRNA. Finally, we show that neither GATA4 nor HNF1α mediates the glucocorticoid-induced precocious maturation of the intestine, but rather are downstream targets of this process. Together, these data demonstrate that specific intestinal genes have differential requirements for GATA4 and HNF1α that are dependent on the developmental time-frame in which they are expressed.

INTRODUCTION

The mature mammalian small intestine is lined by a continuously renewing epithelium that is established through a series of programmed developmental transitions.[1] In mice, beginning on embryonic day (E) 8.5, anterior and posterior invaginations of the visceral endoderm occur that eventually fuse forming a primitive gut tube. Between E9.5 and E14.5, the gut tube undergoes considerable longitudinal growth, and interactions between visceral endoderm and mesoderm result in organ specification. Between E14.5 and E17.5, the process of cytodifferentiation occurs in which the mouse gut endoderm lining the presumptive small intestine is transformed from an undifferentiated, stratified epithelium to a highly differentiated columnar epithelium with villus outgrowth. Dividing cells segregate to the intervillus regions whereas differentiated cells migrate up the villi and begin to express proteins that are critical for intestinal function after birth. During the first week of life, crypts of Lieberkühn develop from the flat intervillus regions resulting in the formation of a distinct proliferating compartment, and the differentiated cells on villi express proteins that are critical for the digestion and absorption of nutrients in milk.[2] During the third week of life corresponding to the weaning transition, the proteins expressed on villi undergo a final transition to an adult pattern designed for the efficient digestion and absorption of nutrients in solid foods. Although the precise timing of events in intestinal development differs between rodents and humans, the fundamental mechanisms underlying cytodifferentiation and the control of villus protein expression during postnatal development are thought to be highly conserved.[3] Fundamental insight into these processes is essential for understanding gut function and the processes that fail in intestinal disease, as well as for creating possible avenues for therapeutic intervention.

Lactase-phlorizin hydrolase (LPH), liver fatty acid binding protein (FABP1), and sucrase-isomaltase (SI) are intestinal proteins important for nutrition during different stages of development, and are also established markers for the transitions that occur in intestinal development.[4-13] LPH and SI are microvillus membrane disaccharidases that hydrolyze milk lactose and α-disaccharides found in solid foods, respectively, whereas FABP1 is a cytoplasmic protein important for intracellular lipid transport. In rodents, LPH and FABP1 are first detected at the beginning of cytodifferentation in preparation for a critical function after birth, continue to be expressed at high levels during the suckling period, and decline during weaning. In contrast, SI is undetectable before weaning and increases to adult levels during weaning. Thus, LPH and FABP1 are markers of cytodifferentiation during fetal development, whereas LPH, FABP1, and SI are indicators of the well-orchestrated patterns of absorptive enterocyte gene expression that occur during postnatal development.

Although much is known about the patterns of *Lph, Fabp1*, and *Si* gene expression during development, the mechanisms underlying these patterns remain to be fully elucidated. Transgenic studies have shown that the 5'-flanking regions of *Lph, Fabp1*, and *Si* direct appropriate tissue, cell-type, and temporal patterns of expression,[10, 14-18] and highly conserved transcription factor

binding sites in the proximal promoters of *Lph*, *Fabp1*, and *Si* have been identified for the GATA family of zinc finger transcription factors, and the hepatocyte nuclear factor-1 (HNF1) and caudal (CDX) families of homeodomain proteins.[19-31] GATA4 and HNF1α are the predominant members of their respective families in nuclear extracts from mouse intestinal epithelial cells that bind to the *Lph* and *Si* promoters,[19, 32, 33] and both GATA4 and HNF1α activate the *Lph, Fabp1*, and *Si* promoters in cell culture over-expression experiments.[19-22, 24, 27-29, 33] GATA4 and HNF1α physically associate and synergistically activate the *Lph*, *Fabp1*, and *Si* genes[19, 22, 33] through an evolutionarily conserved pathway,[33, 34] and we have postulated that this interaction is a means to achieve high levels of intestine-specific gene expression in vivo.[5]

We recently investigated the importance of GATA4 and HNF1α in vivo for intestinal gene expression using gene inactivation approaches.[32, 35] Inactivation of *Gata4* in adult mouse jejunum produces a shift to an ileal-like phenotype, but no obvious consequences in weight, behaviour, skin, or general physiology.[35] Germline *Hnf1α* knockout mice survive into adulthood and demonstrate sterility, diabetes, delayed growth rate, and liver dysfunction.[36] In both models, *Lph* and *Fabp1* mRNA abundances in adult jejunum were reduced ~90%, whereas that of *Si* was surprisingly not affected by the inactivation of either *Gata4* or *Hnf1α*.[32, 35] These data thus indicate that, in adult mouse intestine, both GATA4 and HNF1α are necessary for the expression of the *Lph* and *Fabp1* genes consistent with our model of co-regulation, but are dispensable for *Si* gene expression. The goal of the present study is to use these models to define the requirement of GATA4 and HNF1α for the regulation of *Lph, Fabp1,* and *Si* gene expression in the developing mouse small intestine, specifically focusing on cytodifferentiation and the weaning transition.

MATERIALS AND METHODS

Mice

All animal studies were performed under protocols approved by the Institutional Animal Care and Use Committee of Children's Hospital Boston. Mice were housed in the Animal Research at Children's Hospital facility under standard conditions with 12 h light/dark cycles and were given food and water ad libitum.

Two gene inactivation models were used, including an inducible, intestine-specific *Gata4* inactivation model[35] and a germline *Hnf1α* null model,[32, 36] all in a C56BL/6 background. To inactivate *Gata4* in the small intestinal epithelium, *Gata4*$^{flox/flox}$ mice[37] were crossed with transgenic Villin-*Cre*ER^{T2} mice[38] to generate *Gata4*$^{flox/flox}$, Villin-*Cre*ER^{T2} positive (Cre+) study animals (mutant) and *Gata4*$^{flox/flox}$, Villin-*Cre*ER^{T2} negative (Cre-) controls. To inactivate GATA4 in the intestine, tamoxifen (1 mg/20g BW, Sigma-Aldrich Chemical Co., St. Louis, MO) was administered to timed-pregnant females for 5 consecutive days beginning at E12.5, or for 4 consecutive days beginning at postnatal day (P) 7, as described.[39] *Gata4* mutant mice produce a truncated, transcriptionally inactive form of GATA4 that is capable of site-specific binding to DNA elements, and thus has the

potential for dominant negative activity in vivo.[35] *Hnf1α*-/- mice survive into adulthood, but are sterile, requiring mating of *Hnf1α*+/- parents to generate both null and wild-type study animals.[36] All mice were genotyped using PCR on tail DNA as previously described.[32]

Mice were sacrificed for study at various time-points beginning at E13.5 and extending throughout postnatal development. Study mice or pregnant females were anesthetized with avertin anesthesia (2,2,2-Tribromoethanol, 240 mg/kg BW, Sigma) prior to dissection. For fetal mice, embryos were removed from the mother, transferred to a Petri dish containing 1X PBS, and tissue was isolated using a dissecting microscope. For postnatal mice, tissue was extracted through a midline incision and transferred to a glass plate on a bed of wet ice. All tissues were collected between 1300 and 1600 hr to avoid any fluctuations in gene expression due to circadian cycles.[40]

RNA isolation

RNA was isolated from snap frozen fetal and postnatal mouse tissues. From fetal animals, RNA was isolated from either the entire small intestine, or in the case of selected E17.5 pups, from intestinal segments separated into equal lengths where segment 1 was the most proximal 20%, segment 2 the next 20%, segment 3 the middle 20%, segment 4 the next 20%, and segment 5 the most distal 20% of the small intestine. From postnatal mice, RNA was isolated from 30-50 mg of small intestine (0.5 to 1.0 cm) obtained from the geometric center (segment 3, jejunum). RNA was isolated using the RNeasy™ kit (Qiagen, Valencia, CA). To ensure that all traces of DNA were removed, RNA samples were treated with DNase (DNA-free, Ambion, Austin, TX) for 1 h at 37°C following the manufacturer's instructions. RNA samples were quantified by optical density at A260 nm, and checked for absence of degradation on an agarose gel.

Semi-quantitative and real-time RT-PCR

Semi-quantitative and real-time RT-PCR was conducted as previously described.[32, 33] For both PCR reactions, complementary DNA (cDNA) was synthesized using iScript (BioRad). Primer pairs were designed using Beacon Design software (PREMIER Biosoft International, Palo Alto, CA) and optimized as described.[32, 35] Primer sequences are available upon request. Semi-quantitative RT-PCR experiments were terminated in the linear range of amplification. Real-time RT-PCR was conducted using an iCycler and iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc, Hercules, CA). All real-time RT-PCR data were corrected for *Gapdh* and expressed relative to the calibrator, which was adult jejunal RNA from a single mouse, unless otherwise indicated.

Immunohistochemistry

Immunofluorescence was conducted on mouse tissue as previously described.[35] Following dissection, mouse tissues were immediately immersed in a freshly made solution of buffered 4% paraformaldehyde and incubated for 4 h at 4°C, then resuspended in 70% ethanol overnight. Tissue was embedded in paraffin and 5 μm sections were prepared for immunohistochemistry in

the Department of Pathology, Children's Hospital Boston. Following tissue deparaffinization and rehydration, antigen retrieval was conducted by boiling slides for 10 min in 10mM sodium citrate (pH 6). The slides were then slow-cooled and washed in 1X PBS. After blocking (10% donkey serum in 1X PBS) for 1 h in a humidified chamber, the primary antibody was pipetted onto slides and incubated overnight at 4°C. After washing, the fluorescent secondary antibody was pipetted onto slides and incubated for 4 h at room temperature. Due to cross-reactivity, sequential addition of the two secondary antibodies with extensive washing in between was necessary for GATA4-HNF1α co-immunofluorescence experiments.

The primary antibodies used were goat anti-HNF1α (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-GATA4 (1:100; Santa Cruz), goat anti-GATA4 (1:400; Santa Cruz), rabbit anti-CDX2 (1:500; gift from D. Silberg, University of Pensylvania), rabbit anti-LPH (1:500; gift from K-Y. Yeh, Louisiana State University), rabbit anti-FABP1 (1:1000; gift from J. Gordon, Washington University), and rabbit anti-SI (1:500; gift from K-Y. Yeh, Louisiana State University). The secondary antibodies used were Alexa Fluor 594 donkey anti-goat IgG, Alexa Fluor 488 donkey anti-rabbit IgG, and Alexa Fluor 488 goat anti-mouse IgG, (1:500, Molecular Probes). In most experiments, a solution containing 4',6-diamino-2-phenylindole dihydrochloride (DAPI) nucleic acid stain (2ug/ml, Molecular Probes) in PBS was added to reveal the nuclei.

Isolation of nuclear and non-nuclear extracts

Nuclear extracts were isolated as previously described[33] from pooled mucosal scrapings of 4 cm segments at the geometric center of the small intestine (mid-jejunum) from P4, P7, P14, P21, P28, and adult (6-12 wk) mice. In selected experiments, nuclear and non-nuclear fractions were isolated similarly from P10 mice. The epithelial scrapings were resuspended in hypotonic buffer (10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, protease inhibitor cocktail, 1.0 mM PMSF and 1.0 mM DTT) and centrifuged at 5K rpm for 5 min at 4°C. The cell pellet was resuspended in hypotonic buffer, incubated on ice for 5 min and homogenized in a pre-cooled Dounce homogenizer with 20 strokes using the loose pestle. After centrifuging at 8K rpm for 5 min at 4°C, the supernatant in selected experiments was saved (-70°C) as the non-nuclear fraction. The nuclei were resuspended in low salt buffer (20mM Hepes (pH 7.9), 20 mM KCl, 1.5 mM $MgCl_2$, 25% glycerol, protease inhibitor cocktail, 1.0 mM PMSF and 1.0 mM DTT) followed by the slow addition of high salt buffer (20 mM Hepes (pH 7.9), 1.2 M KCl, 1.5 mM $MgCl_2$, 25% glycerol, protease inhibitor cocktail, 1.0 mM PMSF and 1.0 mM DTT). After extracting at 4°C for 30 min with vigorous mixing every 5 min, the sample was centrifuged at 14K rpm for 15 min at 4°C and the supernatant was saved as the nuclear fraction (-70°C).

Western blotting

Western blot analysis was performed as described previously[32] using 20-80 μg of nuclear or non-nuclear extracts. The primary antibodies included affinity-purified goat polyclonal antibodies for GATA4 or HNF1α (Santa Cruz), a mouse monoclonal antibody for GATA4 (Santa Cruz), or rab-

bit polyclonal antibodies for FABP1 (gift of J. Gordon, Washington University). All blots were stripped and re-probed with anti-mouse β-actin.

EMSA

EMSAs were performed using labelled, double-stranded oligonucleotides containing well characterized binding sites for GATA or HNF1 families of transcription factors, as described previously (Krasinski et al., 2001). These included the GATA binding site present in the Xenopus *Fabp1* promoter (X-GATA, 5'-GGAGATCCCTGTACAGATATGGGGAGAC-3') (Gao et al., 1998), and the HNF1 binding site present in the rat β-fibinogen promoter (β-Fib, 5'-CAAACTGTCAAATATTA-ACTAAAGGGAG-3').[4] Supershift EMSAs were conducted using affinity-purified goat polyclonal antibodies for GATA4 or HNF1α (Santa Cruz).

Dexamethasone treatment

To investigate the role of GATA4 and HNF1α in hormonally induced precocious weaning, a model was used in which wild-type, *Hnf1α*-/- and *Gata4* mutant mice were treated with dexamethasone (Sigma-Aldrich) at P10 essentially as described.[6] Dexamethasone was injected intraperitoneally at 1.0 μg/g body weight. Negative controls included littermates injected with vehicle (0.8% ethanol in 1X PBS). After 4 or 24 h, mice were sacrificed and the jejuna (segment 3) were collected for the isolation of RNA and nuclear extracts as well as for sectioning.

Statistical analyses

Statistically significant differences were determined by the Student's t-test or analysis of variance followed by the Tukey-Kramer multiple comparison test.

RESULTS

GATA4 and HNF1α differentially regulate *Lph* and *Fabp1* gene expression during cytodifferentiation

The mouse intestinal epithelium undergoes rapid cytodifferentiation beginning at E14.5 resulting in the formation of nascent villi and synthesis of intestinal differentiation markers by E17.5. The mRNAs for the markers of cytodifferentiation, *Lph* and *Fabp1*[4, 7, 11] were not detectable by real-time RT-PCR in whole intestine at E13.5 or E14.5, were just detectable at E15.5, and reached their highest levels by E17.5 (Figure 1A). *Lph* and *Fabp1* mRNAs were highest throughout the proximal half of the small intestine (segments 1-3) and declined to nearly undetectable levels in the distal ileum (segment 5), as determined by semi-quantitative RT-PCR (Figure 1B). These data confirm that *Lph* and *Fabp1* are markers for cytodifferentiation of the mouse midgut.

To begin to understand the regulation of target genes by GATA4 and HNF1α during cytodifferentiation, the expression patterns of these transcription factors during this time-frame were

determined. *Gata4* and *Hnf1α* mRNAs were expressed throughout cytodifferentiation, with the highest levels occurring at E17.5 (Figure 2A). Along the cephalo-caudal axis at E17.5, *Gata4* mRNA displayed a declining proximal-to-distal gradient being nearly undetectable in the most distal segment, whereas *Hnf1α* mRNA was nearly undetectable in the most proximal segment, and demonstrated a generally increasing proximal-to-distal gradient, as shown by semi-quantitative RT-PCR (Figure 2B). GATA4 and HNF1α protein were co-localized in epithelial cells of the E13.5 midgut (Figure 2C-E), and on nascent villi and in intervillus regions of the E16.5 (Figure 2F-G) and E17.5 (data not shown) midgut. GATA4 and HNF1α were specifically localized to the nucleus at these ages, as determined by co-staining with DAPI (data not shown). Together, these data demonstrate a topographic basis for possible co-regulation by GATA4 and HNF1α in the midgut during cytodifferentiation.

Inactivation of *Gata4* or *Hnf1α* in the jejunum of adult mice results in an attenuation (~90% reduction) of both *Lph* and *Fabp1* gene expression).[32, 35] To define the importance of GATA4 or HNF1α for *Lph* and *Fabp1* gene expression during cytodifferentiation, we quantified the expression of *Lph* and *Fabp1* mRNAs in our knockout models during this time-frame. To inactivate *Gata4* in the midgut, pregnant mothers carrying *Gata4*$^{flox/flox}$ embryos that were either positive (mutant) or negative (control) for the Villin-*Cre*ER^{T2} transgene, were treated with 5 daily doses of tamoxifen beginning when pups were E12.5 (Figure 3A). At E17.5, *Gata4* was specifically inactivated in the midgut of *Gata4* mutant mice (Figure 3B) verifying the model. Body size, gross intestinal structure, and overall intestinal histology, as indicated by H&E staining, of *Gata4* mutant mice were indistinguishable from controls. *Lph* mRNA abundance in the midgut of *Gata4* mutant mice was also indistinguishable from controls, whereas *Fabp1* mRNA abundance was reduced ~50% (Figure 3C, top). In *Hnf1α*-/- mice, both *Lph* and *Fabp1* mRNA abundances were significantly reduced ($P<0.05$) by ~50% as compared to *Hnf1α*+/+ wild-type controls (Figure 3C, bottom). These data indicate that during cytodifferentiation, GATA4 is dispensable for *Lph* gene expression but partially required for *Fabp1* gene expression, whereas Hnf1α is partially required for both *Lph* and *Fabp1* gene expression.

Nuclear GATA4 and HNF1α are paradoxically reduced before weaning despite high levels of their respective mRNAs

To begin to define the underlying mechanism by which GATA4 and HNF1α differentially regulate intestinal genes during postnatal development, the patterns of expression of *Gata4* and *Hnf1α* mRNAs and proteins were first determined in wild-type mice at P7, P14, P21, and P28. *Gata4* mRNA was highest before weaning, and gradually declined during weaning to significantly lower levels at P21 ($P<0.05$) (Figure 4A, upper). *Hnf1α* mRNA was highest at P7, and was significantly lower at all time-points thereafter (Figure 4A, bottom). These patterns are generally consistent with recently published data.[42]

To determine if the mRNA abundances correlate with nuclear protein levels, Western blot analyses using nuclear extracts from mid-jejunum of wild-type mice were performed. GATA4

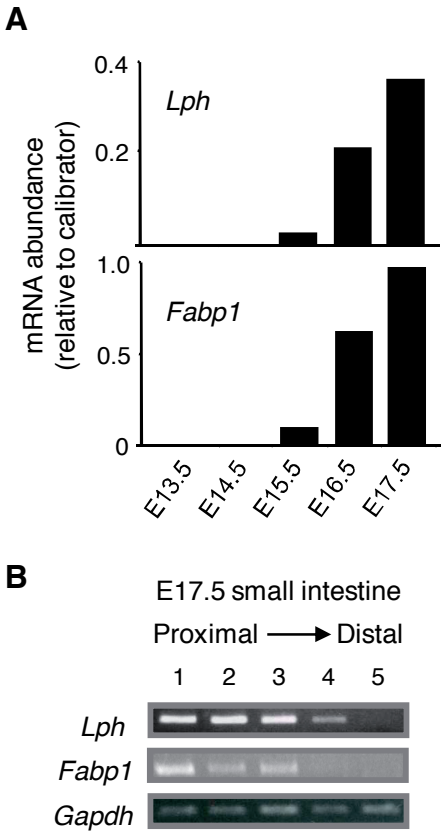

Figure 1. *Lph* and *Fabp1* mRNAs are induced in the mouse small intestine during cytodifferentiation. (A) *Lph* and *Fabp1* mRNAs are first detected in mouse small intestine at E15.5. *Lph* and *Fabp1* mRNAs were quantified by real-time RT-PCR in whole intestine of mouse embryos obtained from timed-pregnant mothers. Data were obtained from a single mouse at each time point. (B) *Lph* and *Fabp1* mRNAs at E17.5 are highly expressed in the proximal half of mouse small intestine and decline distally. RNA was isolated from 5 equidistant segments of a mouse embryo at E17.5 as described in Materials and Methods and the abundances of *Lph* and *Fabp1* mRNAs were determined by semi-quantitative RT-PCR. *Gapdh* is shown as a positive control.

and HNF1α were surprisingly low at P7 and P14, but increased markedly at P21 and P28 (Figure 4B), sharply contrasting with their decreasing mRNA levels during this time interval (see Figure 4A). This was verified using 40-80 μg of nuclear extracts isolated additionally from the jejunum of P4, P7, and P10 mice (Figure 4C). GATA4 was detected at all time points but at much lower levels than in adults, with the lowest level occurring at P7, whereas HNF1α could not be detected at P4 and P7. To determine if GATA4 and HNF1α are localized outside of the nucleus before weaning, Western analyses were performed on non-nuclear fractions isolated from jejunal enterocytes at P10. As shown in Figure 4D, neither GATA4 nor HNF1α was detected in the non-nuclear fractions at this age. As controls, both GATA4 and HNF1α were readily detected in nuclear extracts of

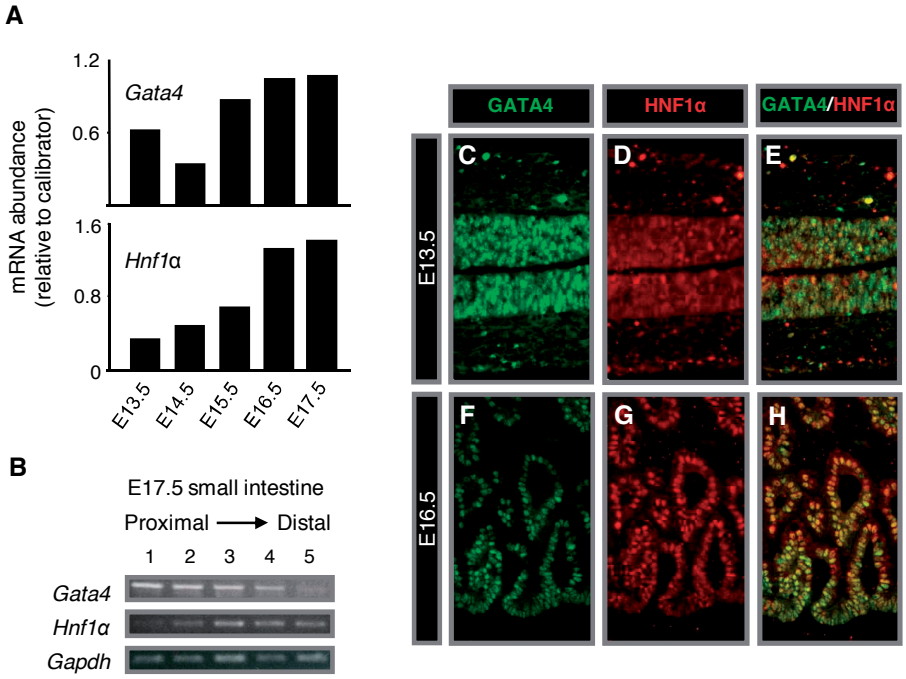

Figure 2. GATA4 and HNF1α are co-expressed in the intestinal epithelial cells of the presumptive small intestine during cytodifferentiation. (A) *Gata4* and *Hnf1α* mRNAs demonstrate an increasing pattern during cytodifferentiation. *Gata4* and *Hnf1α* mRNAs were quantified by real-time RT-PCR in whole intestine of mouse embryos obtained from timed-pregnant mothers. Data were obtained from a single mouse at each age group. (B) *Gata4* mRNA at E17.5 is highly expressed in the proximal half of mouse small intestine and declines distally, whereas *Hnf1α* mRNA is low in proximal intestine and increases distally. RNA was isolated from 5 equidistant segments of a mouse embryo at E17.5 as described in Materials and Methods, and the abundance of *Gata4* and *Hnf1α* mRNA was determined by semi-quantitative RT-PCR. (C-H) GATA4 and HNF1α are co-expressed in epithelial cells of presumptive small intestine at E13.5 (C-E), and E16.5 (segment 3) (F-H). Immunofluorescence was conducted using mouse anti-GATA4 and goat anti-HNF1α, as indicated. Co-localization of GATA4 and HNF1α is indicated by merged photomicrographs as indicated (E, H).

wild-type jejunum from adult mice, but not in the mature jejunum from the respective knockout models. The relative abundance of FABP1 in the non-nuclear fraction verifies the enrichment of cytoplasmic protein in this fraction. The absence of GATA4 or HNF1α in non-nuclear fractions before weaning was replicated on pooled samples of P7 mouse jejunum. Nuclear extracts from both P7 (Figure 4E) and P10 (data not shown) were further tested by EMSAs using well characterized DNA binding sites for GATA[43] and HNF1[4] transcription factors. Although specific complexes (filled arrowheads) that supershifted (open arrowheads) with specific antibodies were readily detected in nuclear extracts isolated from P28 mouse jejunum, complexes were barely detectable or

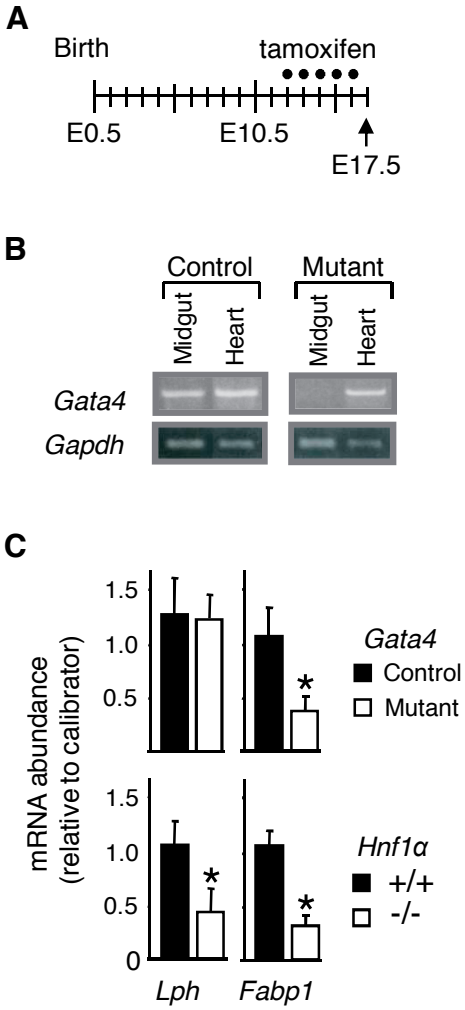

Figure 3. GATA4 and HNF1α are differentially required for *Lph* and *Fabp1* gene expression during cytodifferentiation. (A) Schematic representation showing that timed-pregnant mothers were treated with 5 daily injections of tamoxifen (filled circles) beginning at E12.5. Mice were sacrificed for analysis at E17.5 (arrow). (B) GATA4 is specifically inactivated in the midgut of *Gata4* mutant mice at E17.5. Semi-quantitative RT-PCR for *Gata4* was conducted on RNA isolated from the midgut (segment 3) and heart of a representative control and *Gata4* mutant mouse as indicated. (C) GATA4 is dispensable for *Lph* gene expression but partially required for *Fabp1* gene expression, whereas HNF1α is partially required for both *Lph* and *Fabp1* gene expression. Real-time RT-PCR for *Lph* (left panels) and *Fabp1* (right panels) mRNA was conducted on RNA isolated from E17.5 midgut (segment 3) of control and *Gata4* mutant mice (top panels), and wild-type (+/+) and *Hnf1α* null (-/-) mice (bottom panels). The calibrator was midgut RNA from one of the control mice. Data are mean ± SD of n = 3-5 mice. *P<0.05 as compared to control or wild-type (+/+) mice.

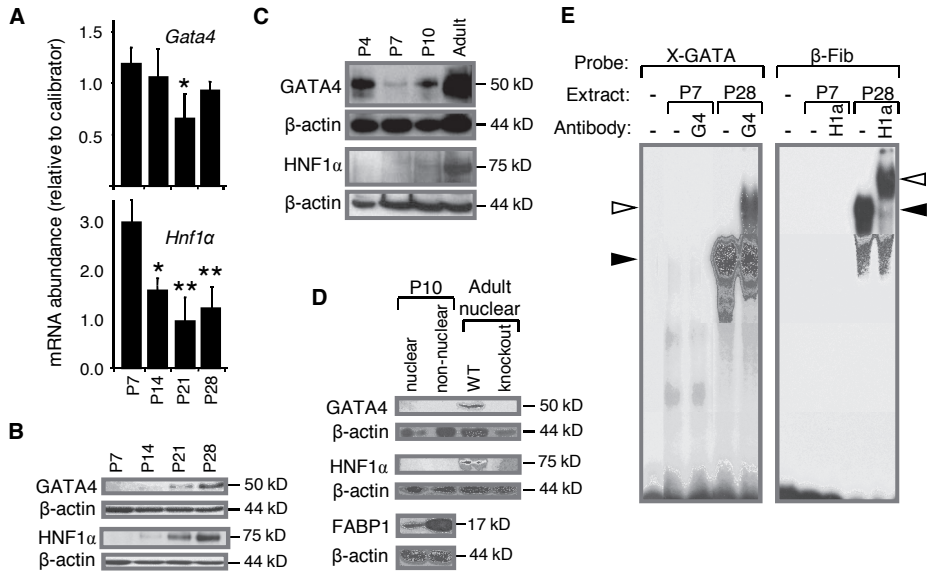

Figure 4. Nuclear GATA4 and HNF1α are paradoxically reduced before weaning despite high levels of their respective mRNAs. (A) *Gata4* and *Hnf1α* mRNA abundances decrease during the weaning transition. *Gata4* and *Hnf1α* mRNAs were quantified in wild-type jejunum (segment 3) at P7, P14, P21, and P28 by real-time RT-PCR. Data are mean ± SD of n = 3-5 mice. *P<0.05, as compared to P7. (B) GATA4 and HNF1α protein levels increase during the weaning transition. GATA4 and HNF1α were quantified in nuclear extracts isolated from wild-type jejunum at P7, P14, P21, and P28 by Western analysis using polyclonal goat antibodies for GATA4 and HNF1α. Blots were re-probed with a β-actin antibody to demonstrate similar protein loading in each lane. (C) GATA4 and HNF1α are reduced before weaning as compared to adults. GATA4 and HNF1α were quantified in nuclear extracts from wild-type jejunum at P4, P7, P10 and adults by Western analysis using a mouse monoclonal GATA4 antibody and a goat polyclonal HNF1α antibody. Forty and 80 μg of protein were used for GATA4 and HNF1α analyses, respectively. (D) GATA4 and HNF1α were not detected in non-nuclear fractions of P10 mouse jejunum. GATA4 and HNF1α were quantified in nuclear and non-nuclear extracts isolated from wild-type jejunum at P10, and in nuclear extracts from wild-type knockout adult jejunum by Western analysis using polyclonal goat antibodies for GATA4 and HNF1α. FABP1 immunoblot was conducted to verify the enrichment of cytoplasmic proteins in the non-nuclear fractions. (E) GATA and HNF1 binding activity was not detected in nuclear extracts from P7 mouse jejunum. Using well characterized sites for GATA (X-GATA) and HNF1 (β-Fib) binding, EMSAs were conducted using nuclear extracts obtained from P7 and P28 mouse jejunum. Specific complexes (filled arrowhead) that supershifted (open arrowhead) with specific GATA4 (G4) or HNF1α (H1a) antibodies were found only for extracts from P28 mice.

undetectable in nuclear extracts from P7 jejunum. Together, these data indicate that GATA4 and HNF1α are present at very low levels in the epithelial nuclei before weaning.

GATA4 and HNF1α are readily detected in the nuclei of villi and intervillus regions of embryonic intestine by immunofluorescence (see Figure 2). However, just after birth (P1), GATA4 was expressed only in the intervillus regions, not on villi (Figure 5A, B), and HNF1α could not be detected in either compartment (Figure 5C). From P4 to P10, neither GATA4 nor HNF1α

were detected, as exemplified by immunofluorescence of P7 intestine (Figure 5D-G). As a positive control, all sections during this time interval positively stained for CDX2 (Figure 5H, I), an intestinal nuclear transcription factor that is expressed in the intestinal epithelium throughout development.[44] At P14, GATA4 was detected only in the nuclei of cells in the crypts and lower villi (Figure 5J, K), whereas HNF1α was not detected (data not shown). By P21, GATA4 and HNF1α were co-expressed throughout the villus epithelium (Figure 5L-M), as in adults. Together, these studies demonstrate that both the *Gata4* and *Hnf1α* genes are expressed at high levels throughout postnatal development as indicated by their high levels of mRNA (see Figure 4A), but their protein products are expressed at low levels during suckling.

GATA4 and HNF1α differentially regulate target gene expression during postnatal development

During weaning, which in mice occurs throughout the third week of life, the proteins expressed on villi undergo a final transition from a suckling pattern optimized for the synthesis of enzymes important for the digestion of nutrients in milk, to an adult pattern designed for the efficient digestion and absorption of nutrients in solid foods.[2] After birth and throughout suckling, *Lph* and *Fabp1* are highly expressed, and decline during weaning, whereas *Si* is low before weaning and increases during weaning. To define the importance of GATA4 or HNF1α for *Lph*, *Fabp1*, and *Si* gene expression during weaning, we quantified the mRNAs for these genes in our knockout models during this time-frame. To inactivate *Gata4* in jejunum, we employed a time-course essentially as described previously[39] (Figure 6A) whereby 7-day old mice were treated for 4 consecutive days with a single daily injection of tamoxifen. Mice were sacrificed for study at P10 (pre-weaning), P20 (mid-weaning) and P30 (post-weaning) (Figure 6A, arrows). *Gata4* was expressed normally in heart, liver and stomach (data not shown), but was absent in the jejunum at all ages (Figure 6B), verifying the *Gata4* inactivation model for the study of postnatal development.

Growth rate and overall intestinal structure and histology (data not shown) in *Gata4* mutant mice were indistinguishable from controls throughout the weaning transition. Analysis of *Lph* gene expression in these mice revealed that *Lph* mRNA abundance in mid-jejunum at P10 and P20 of *Gata4* mutant mice was similar to that in control mice, but at P30 was <10% of that in control mice ($P<0.05$, Figure 7A, top), a difference that is similar to that in adult mice.[35] *Fabp1* mRNA levels were reduced by the inactivation of *Gata4* ~50% at P10 ($P<0.05$, Figure 7A, middle), similar to that observed at E17.5 (see Figure 3C). *Fabp1* mRNA abundance in *Gata4* mutant mice at P20 and P30 was <10% of that in control mice ($P<0.05$, Figure 5A, middle), similar to that in adult mice.[35] *SI* mRNA levels revealed an expected increase during weaning, but no difference between control and *Gata4* mutant mice at any postnatal time-point (Figure 7A, bottom).

Immunofluorescence for LPH, FABP1, and SI in control and *Gata4* mutant mice during weaning generally followed the results of their respective mRNA abundances. LPH immunofloure-sence was specific to the microvillus membrane in the P10 jejunum (Figure 7B), and was not affected by the inactivation of *Gata4* (Figure 7C), consistent with mRNA levels. LPH immuno-

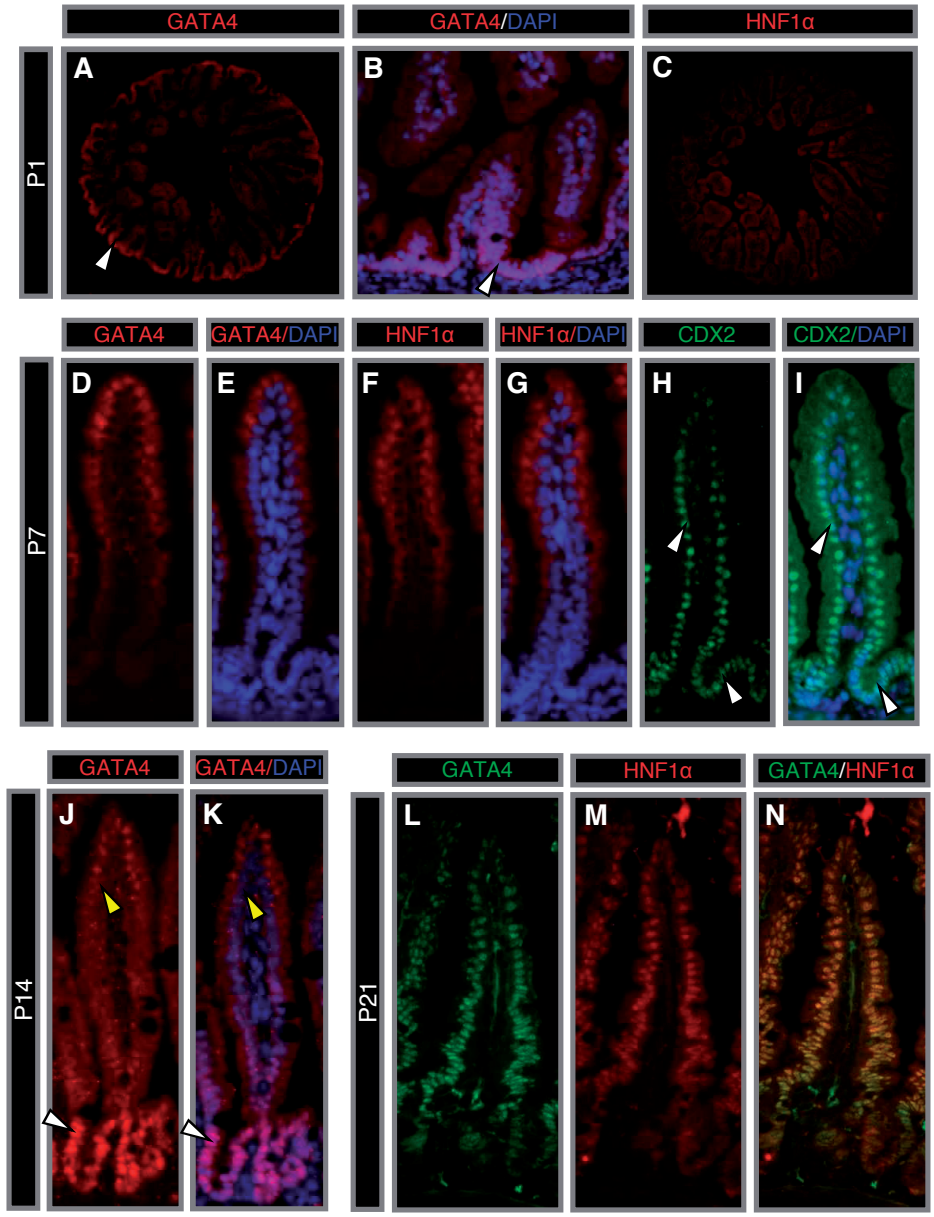

Figure 5. GATA4 and HNF1α expression is attenuated in jejunum before weaning. (A-C) Immunofluorescence in P1 jejunum showing GATA4 in the nuclei of the intervillus regions, but not on villi (A, B). HNF1α was not detected in either compartment at this age (C). (D-I) Immunofluorescence in P7 jejunum reveals an inability to detect either GATA4 (D, E) or HNF1α (F, G). CDX2 is readily detected in the nuclei throughout the crypt and villus epithelium (H, I). (J, K) Immunofluorescence in P14 jejunum showing GATA4 in crypt nuclei, but not on villi. (L-M) Co-immunofluorescence showing co-expression of GATA4 and HNF1α in the P21 jejunum. White arrowheads show specific nuclear immunofluorescence and yellow arrowheads indicate the absence of specific nuclear immunofluorescence.

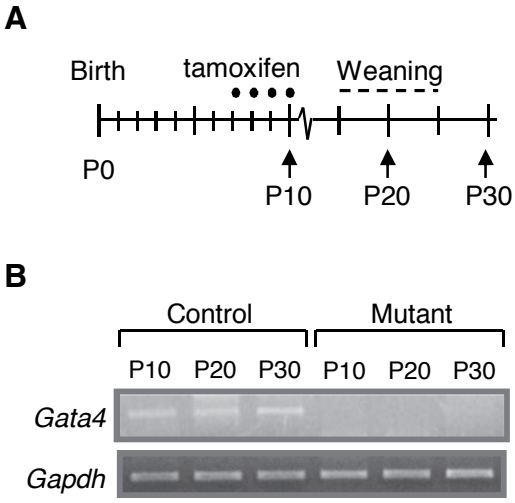

Figure 6. *Gata4* is inducibly inactivated in the mouse jejunum during postnatal development. (A) Schematic representation showing that postnatal mice were treated with 4 daily injections of tamoxifen (filled circles) beginning at P7. Mice were sacrificed for analysis at P10, P20, and P30 (arrows). The weaning transition is indicated by the dotted line. (B) *Gata4* is inactivated in the jejunum of *Gata4* mutant mice during postnatal development. Semi-quantitative RT-PCR for *Gata4* mRNA was conducted on RNA obtained from the jejunum of representative control and *Gata4* mutant mice at P10, P20, and P30. *Gapdh* is shown as a positive control.

fluorescence was also present in the jejunum of P30 control mice (Figure 7D), but absent in the *Gata4* mutant mice (Figure 7E), which is also consistent with mRNA levels. FABP1 immunofluorescence in the cytoplasm of villus enterocytes was reduced by the inactivation of *Gata4* at both P10 and P30 (Figure 7F-I), consistent with its mRNA levels. SI was not detected at P10 in either control or *Gata4* mutant mice (Figure 7J, K), and was localized to the microvillus membrane at P30 with no apparent difference in intensity between control and *Gata4* mutant mice (Figure 7L, M), consistent with mRNA levels. These data demonstrate that GATA4 is: not required for *Lph* gene expression before weaning, but indispensable after weaning; at least partially required for *Fabp1* gene expression throughout development; and not required for *Si* gene expression at any time during development.

In *Hnf1α*-/- mice, *LphH* mRNA abundance was ~50% of that in wild-type jejunum at P7, P14, and P21, similar to that at E17.5, but was <10% of that in wild-type jejunum at P28 (Figure 8A, top), similar to that in adult *Hnf1α* null mice.[32] *Fabp1* mRNA was reduced ~50% in the *Hnf1α* null mice at P7 and P14, similar to that at E17.5 (see Figure 3C), and was barely detectable at P21 and P28 (Figure 8A, middle), similar to that in adults.[32] *Si* mRNA increased during weaning in *Hnf1α*+/+ and *Hnf1α*-/- mice with no significant difference between the two groups (Figure 8A, bottom).

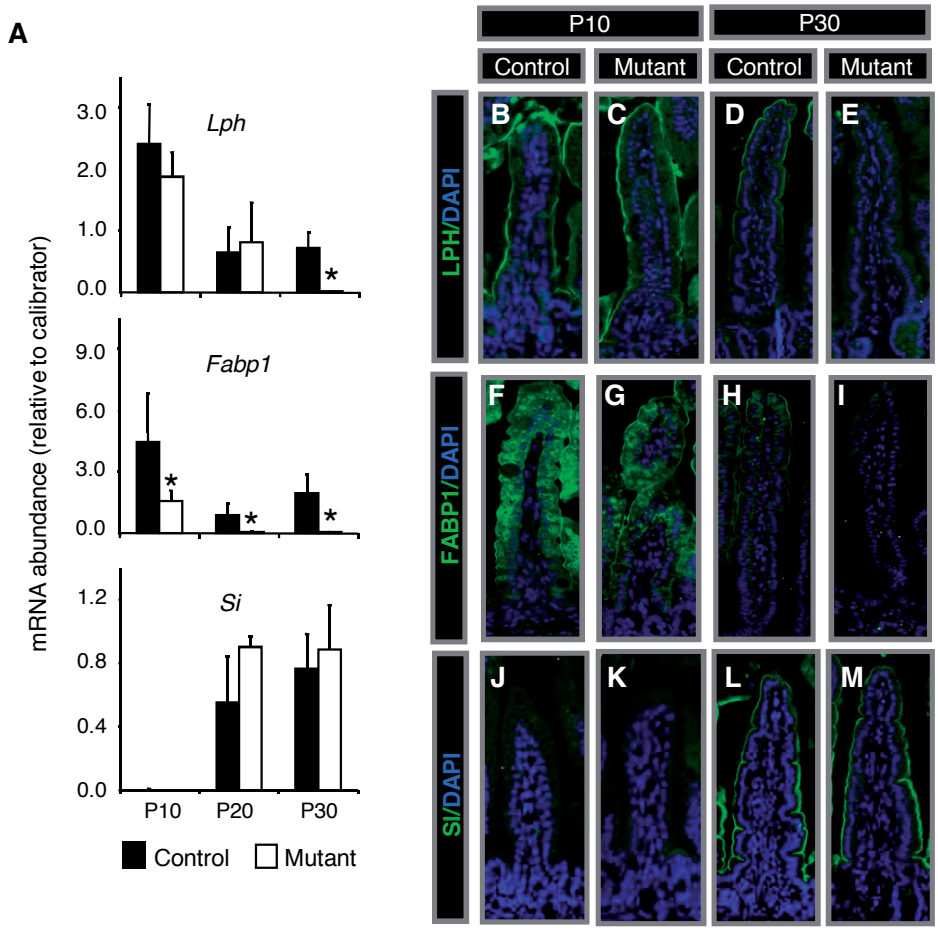

Figure 7. Inactivation of *Gata4* in jejunum reveals gene-specific, developmental regulation. (A) *Lph*, *Fabp1*, and *Si* mRNA abundances are differentially affected by the inactivation of *Gata4* during development. Real-time RT-PCR for *Lph* (top), *Fabp1* (middle) and *Si* (bottom) mRNAs were conducted on RNA isolated from jejunum (segment 3) of control and *Gata4* mutant mice at P10, P20 and P30. Data are mean ± SD of n = 3-5 mice. *P<0.05, as compared to controls. (B-M) Immunofluorescence for LPH (B-E), FABP1 (F-I), and SI (J-M) in P10 control (B, F, J), P10 *Gata4* mutant (C, G, K), P30 control (D, H, L), and P30 *Gata4* mutant (E, I, M) mice.

LPH immunofluorescence was most intense in the P7 jejunum of wild-type mice (Figure 8B), and was consistently less intense in the P7 jejunum of *Hnf1α* null mice (n=3, Figure 8C), in agreement with the decrease in *Lph* mRNA levels at this age. LPH immunofluorescence was present in the P28 jejunum of wild-type mice (Figure 8D), but was not detectable in the jejunum of P28 *Hnf1α* null mice (Figure 8E), again consistent with mRNA levels. FABP1 immunofluorescence was reduced in *Hnf1α* null mice at both P7 and P28, consistent with its mRNA levels (Figure 8F-I). SI immunofluorescence was not detected in either control or *Hnf1α* null mice at P7 (Figure 8J,

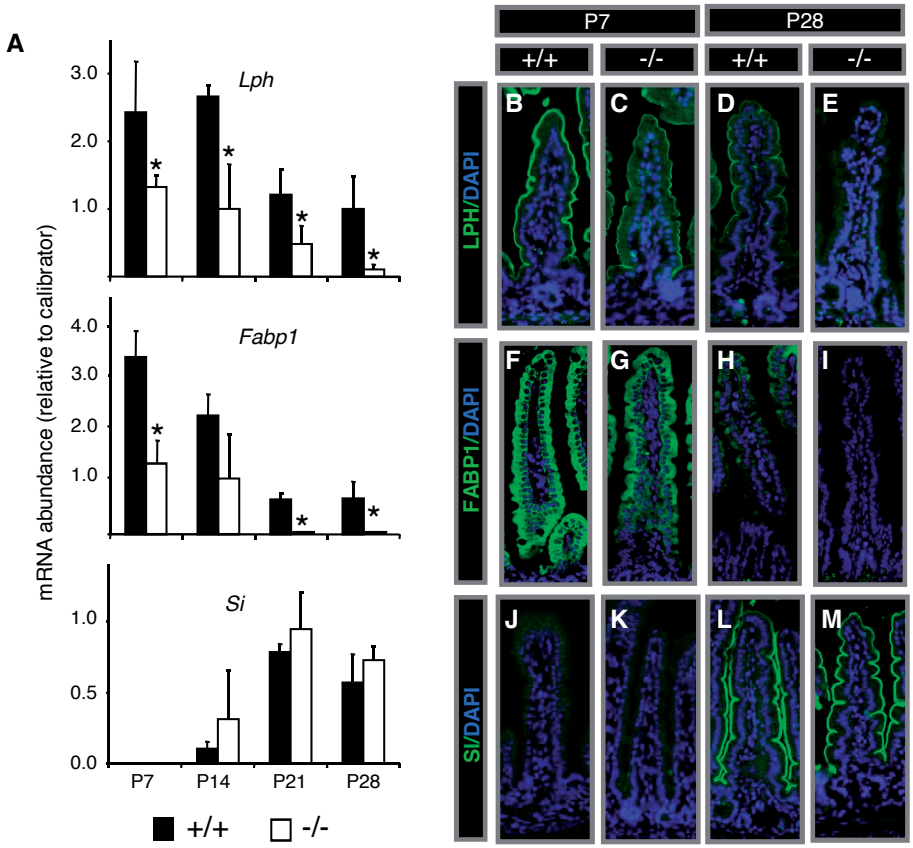

Figure 8. Null expression of *Hnf1α* demonstrates gene-specific, developmental regulation. (A) *Lph*, *Fabp1*, and *Si* mRNA abundances are differentially affected by null expression of *Hnf1α* during development. Real-time RT-PCR for *Lph* (top), *Fabp1* (middle) and *Si* (bottom) mRNAs were conducted on RNA isolated from jejunum (segment 3) of *Hnf1α*+/+ and *Hnf1α*-/- mice at P7, P14, P21, and P28. Data are mean ± SD of n = 3-5 mice. *P<0.05, as compared to controls. (B-M) Immunofluorescence for LPH (B-E), FABP1 (F-I), and SI (J-M) in P7 *Hnf1α*+/+ (B, F, J), P7 *Hnf1α*-/- (C, G, K), P28 *Hnf1α*+/+ (D, H, L), and P28 *Hnf1α*-/- (E, I, M).

K), and was similarly intense in the jejunum of both mice at P28 (Figure 8L, M), correlating with its mRNA levels. These data indicate that HNF1α, although indispensable for *Lph* and *Fabp1* gene expression after weaning,[32] is only partially required before weaning. These data also show that HNF1α is not required for the endogenous increase in *Si* gene expression during weaning.

GATA4 and HNF1α do not mediate the precocious weaning induced by glucocorticoids

Glucocorticoids are known to induce maturation of the small intestine resulting in the precocious induction of intestinal enzymes such as SI.[6] However, because this induction is characterized by an 8 h lag, it is thought to be a secondary effect. The primary response is likely mediated by intestinal transcription factors and GATA factors have been implicated.[45] To define the possible role of GATA4 as well as HNF1α in mediating the glucocorticoid response in preweaning mice, we characterized the dexamethasone-induced response in the context of null intestinal expression of *Gata4* or *Hnf1α*. As shown in Figure 9A (top), *Si* mRNA abundance was similarly induced ~80-fold 24 h after dexamethasone administration in wild-type, *Gata4* mutant, and *Hnf1α*-/- mice indicating that neither GATA4 nor HNF1α is necessary to mediate the dexamethasone response on SI in vivo. *Lph* mRNA abundance was not affected by dexamethasone in wild-type mice, but was significantly reduced by dexamethasone in both *Gata4* mutant and *Hnf1α*-/- mice (Figure 9A, bottom). These data indicate that dexamethasone induces a process in which GATA4 and HNF1α become regulatory for *Lph* gene expression, as in the post-weaning situation. In wild-type mice, the mRNAs for both *Gata4* and *Hnf1α* were significantly reduced by dexamethasone, both at 4 h (data not shown) and 24 h (Figure 9B) after dexamethasone treatment, similar to that which occurs after weaning. Taken together, these data indicate that GATA4 and HNF1α are not required for the precocious maturation process induced by glucocorticoids, but are likely downstream targets of this process.

DISCUSSION

The establishment of a fully functioning mature mammalian gut is the result of a series of ordered developmental transitions.[1] The terminal differentiation phases of intestinal development, characterized in part by the expression of proteins necessary for the digestion and absorption of nutrients, occur during cytodifferentiation and the weaning transition.[2] LPH, FABP1 and SI are well characterized markers of these transitions,[4-10, 12] and the GATA and HNF1 families of transcription factors have been implicated in their regulation.[19-24, 26-29, 31]

In mature jejunum, we have previously shown that GATA4 and HNF1α are necessary for *Lph* and *Fabp1* gene expression, but not for *Si* gene expression.[32, 35] In the present study, we found that the regulation of these target genes by GATA4 and HNF1α during development differs from that in adults (Figure 10), in that before weaning, including during cytodifferentiation, GATA4 and HNF1α are either not required, or only partially required for *Lph* and *Fabp1* gene expression, contrasting with their indispensability after weaning. The partial GATA4 requirement for *Fabp1* gene expression during cytodifferentiation is consistent with data from E18.5 mosaic *Gata4* knockout mice in which *Fabp1* gene expression is attenuated in intestinal epithelial cells that do not express GATA4 as indicated by in situ hybridization.[22] We also found that GATA4 and HNF1α are not

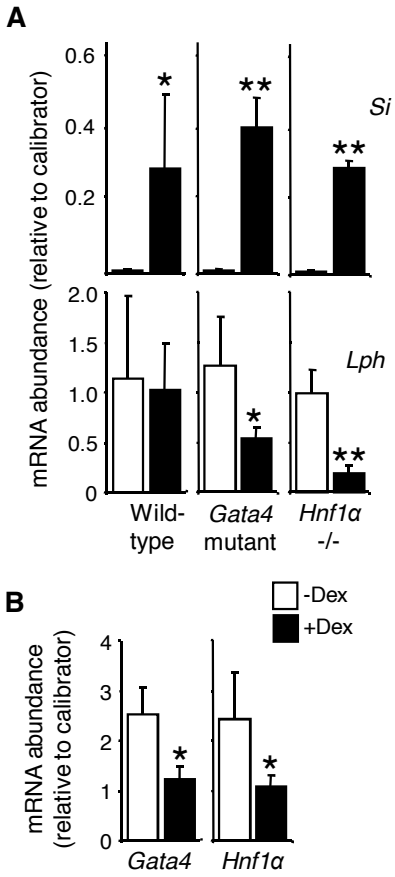

Figure 9. Dexamethasone induces a precocious weaning response in jejunum in the presence or absence of GATA4 or HNF1α. (A) Dexamethasone induces *Si* mRNA levels in preweaning mice independently of GATA4 or HNF1α, but attenuates *Lph* mRNA levels specifically in the absence of GATA4 or HNF1α. Mice were treated with dexamethasone (1 μg/g BW) on P10 and RNA was collected 24 h later from the jejunum of wild-type, *Gata4* mutant, and *Hnf1α*-/- mice. *Si* (top) and *Lph* (bottom) mRNA levels were quantified by real-time RT-PCR in n = 3-5 mice. *P<0.05, **P<0.01 as compared to untreated mice. (B) Dexamethasone attenuates *Gata4* and *Hnf1α* mRNA levels in jejunum of preweaning mice. Wild-type mice were treated with dexamethasone on P10 and RNA was collected 24 h later from the jejunum. *Gata4* and *Hnf1α* mRNA levels were quantified by real-time RT-PCR in n = 3-5 mice. *P<0.05, as compared to untreated mice.

required for *Si* gene expression at any time during development. During the suckling period, we found a surprising and dramatic reduction in GATA4 and HNF1α protein in the nuclei of absorptive enterocytes of the jejunum despite high levels of mRNA. Finally, we show that neither GATA4 nor HNF1α mediate the precocious maturation of the intestine induced by glucocorticoids. Together, these data demonstrate that specific intestinal genes, including *Lph* and *Fabp1*,

have differential requirements for GATA4 and HNF1α that are dependent on the developmental time-frame in which they are expressed.

Our data show that GATA4 or HNF1α are partially required for *Lph* and *Fabp1* gene expression before weaning, which contrasts with their indispensability for *Lph* and *Fabp1* gene expression after weaning. Functionally, LPH is necessary for the digestion of milk lactose, and is thus critical for nutrition during suckling, while FABP1 plays a role in intracellular lipid transport, and is likely important for the transport of the lipid load present in milk. Thus, a plausible hypothesis is that a redundant mechanism for the maintenance of gene expression during this critical developmental time interval is necessary. These data also indicate that other factors are involved in *Lph* and *Fabp1* gene expression before weaning. Candidate transcription factors include GATA5, GATA6, HNF1β, and members of the CDX, HNF3, and C/EBP families, all of which have been previously implicated as activators of *Lph* and/or *Fabp1* gene transcription in vitro.[19, 22-27, 30, 46-48] Additional studies will be necessary to define the requirement in vivo of these transcription factors and/or identify other factors involved in the developmental regulation of *Lph* and *Fabp1* gene expression.

These data also reveal differential mechanisms underlying the regulation of genes whose expression patterns during development are strikingly similar. Both *Lph* and *Fabp1* are highly expressed in more proximal regions of small intestine than distal regions, and at higher levels before weaning than after weaning (Figure 1, 7 and 8, and[4, 5, 7, 8, 10]) and their promoters contain binding sites for similar sets of transcription factors.[21, 27] However, although both are similarly regulated by GATA4 and HNF1α in mature intestine,[32, 35] they are differentially regulated by these two transcription factors before weaning. Both *Lph* and *Fabp1* mRNAs are reduced ~50% in *Hnf1α* null mice before weaning, but only *Fabp1* mRNA is reduced in the *Gata4* mutant mice before weaning. Further, *Fabp1* mRNA abundance is attenuated ~90% by the inactivation of *Gata4* at P20 or *Hnf1α* at P21, similar to that in adult mice, whereas *Lph* mRNA abundance is reduced <50% at these time points, similar to preweaning mice, indicating a differential in the timing of regulation by GATA4 or HNF1α. The differential regulation of *Lph* and *Fabp1* by GATA4 before weaning highlights a target-specific gene regulation during development.

We have previously hypothesized that the induction of *Si* gene expression during postnatal development is regulated by the combinatory effect of a complex of transcription factors including GATA4 and HNF1α.[19, 27] In addition, the abundance of nuclear GATA4 and HNF1α proteins in the jejunum increases during the weaning transition, paralleling *Si* gene expression (Figure 4B and[19, 27]). Despite these compelling data for a combinatory role by *Gata 4* and *Hnf1α* in the activation of *Si* gene expression, we recently reported that the inactivation of GATA4 or HNF1α had no effect on *Si* gene expression in adult mice.[32, 35] Here, we show that the inactivation of *Gata4* or *Hnf1α* had no effect on the initiation of *Si* gene expression during weaning. Together, these data demonstrate that GATA4 and HNF1α are dispensable for *Si* gene expression throughout development. The future challenge, therefore, is to identify transcription factors essential for *Si* gene expression.

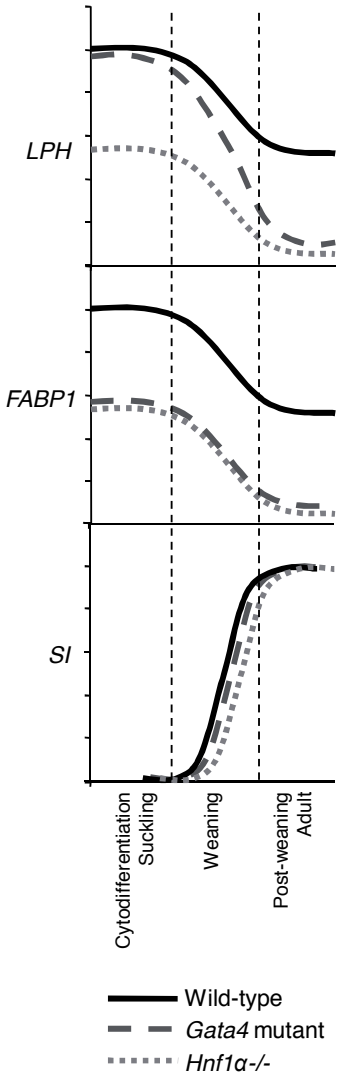

Figure 10. Summary of GATA4 and HNF1α regulation of *Lph*, *Fabp1* and *Si* gene expression during intestinal development. Schematic representation of *Lph* (top), *Fabp1* (middle), and *Si* (bottom) gene expression in wild-type (solid line), *Gata4* mutant (dashed line), and *Hnf1α-/-* (dotted line) mice during development.

In our studies, we identified a paradoxical loss of GATA4 and HNF1α protein in the nuclei of absorptive enterocytes beginning shortly after birth, continuing through the suckling period, and ending during the third week of life when weaning occurs. At P4-P10, GATA4 and HNF1α were greatly reduced in the nuclear fraction of jejunal extracts as determined by Western analysis (Figure 4B-D) and EMSA (Figure 4E), and neither could be detected in the nuclei of villus enterocytes

in the jejunum by immunofluorescence (Figure 5D-I). Interestingly, *Gata4* and *Hnf1*α mRNA abundance remains high during this time interval (Figure 4A) suggesting that the reduction in GATA4 and HNF1α protein is not due to a decrease in transcription rate. We thus believe that either the mRNAs for these proteins are not transcribed, and/or that their translation products are actively catabolized.

Although GATA4 and HNF1α are greatly reduced or absent in the intestinal epithelial nuclei during suckling, they are nevertheless partially required for *Lph* and *Fabp1* gene expression during this time-frame (Figure 7 and 8). One explanation is that at least some GATA4 and HNF1α is normally present in the nucleus, as suggested by Western analysis for GATA4 using 40 μg of protein (Figure 4C). However, HNF1α could not be detected at P4 and P7 on Western blots using 80 μg of protein (Figure 4C), suggesting that HNF1α is not present during this time interval. Thus, a second explanation is that GATA4 and/or HNF1α are required earlier in intestinal development for later expression of putative target genes. While this is a plausible explanation for HNF1α, in which a germline null model was used, it is a less likely explanation for GATA4, which is inducibly inactivated after birth. Precedence for an early developmental requirement for later expression is shown in the liver where embryonic, but not postnatal re-expression of HNF1α is capable of re-activating the silent phenylalanine hydroxylase gene in HNF1α-deficient hepatocytes.[49]

Interestingly, regulation in the knockout models at P7-P14 (Figure 7 and 8), when GATA4 and HNF1α nuclear protein is decreased, is virtually identical to that which occurs at E17.5 (Figure 3), when GATA4 and HNF1α are normally present in epithelial nuclei, suggesting that the loss of nuclear GATA4 and HNF1α after birth is not a critical regulatory mechanism for *Lph* and *Fabp1* gene expression. Thus, it is possible that the process of nuclear GATA4 and HNF1α loss during suckling in mice is a regulatory process, but for other as yet unknown targets of GATA4 and HNF1α.

Glucocorticoids like dexamethasone can induce the precocious maturation of the intestine, but the underlying mechanism has not been fully elucidated.[50, 51] Characteristic of this process is a dramatic increase in *Si* mRNA abundance 24 h after the administration of glucocorticoids.[6] Since the induction in *Si* mRNA is not apparent within the first 8 h,[6] it is thought that the *Si* induction is not a direct response to glucocorticoids, but rather a secondary effect of early response genes on *Si* gene transcription.[50] Recently, Oesterreicher and Henning[45] showed that in 8-day old mice, GATA4 and GATA6 were both induced 4 h after dexamethasone treatment, as shown by supershift EMSAs and Western analysis, suggesting a role for GATA factors in the glucocorticoid-induced maturation of the intestine. To test the hypothesis that GATA4 or HNF1α mediates this process, we conducted dexamethasone-induced precocious maturation experiments in our knockout models. *Si* mRNA was strongly induced in the presence or absence of GATA4 or HNF1α (Figure 9A) indicating that these proteins are not required for mediating the dexamethasone response on *Si*. We next defined the effect of dexamethasone on *Lph* mRNA abundance in our knockout models, and found that *Lph* mRNA was reduced in the *Gata4* mutant and *Hnf1*α-/- mice (Figure 9A), but not in wild-type controls. Our interpretation of these data is that dexamethasone induces

precocious maturation to the point where GATA4 and HNF1α become more regulatory for *Lph* gene expression. We also found a decrease in both *Gata4* and *Hnf1α* mRNA abundance with dexamethasone treatment (Figure 9B), which is also consistent with a maturation of the intestine as *Gata4* and *Hnf1α* mRNAs decline normally during weaning (Figure 4A). Taken together, we believe that neither GATA4 nor HNF1α mediates the glucocorticoid response but rather are downstream targets of this response.

Cre-mediated inactivation of *Gata4* in our current model results in the synthesis of a truncated, trancriptionally inactive form of GATA4 (mutant GATA4) that is missing its N-terminal activation domains, but contains its functional zinc finger region.[35] Since mutant GATA4 continues to bind DNA, it has the potential to act as a dominant-negative GATA factor[35] masking the activity of other enterocyte GATA factors, such as GATA6, which is co-expressed with GATA4 in villus enterocytes.[22, 35, 52] Further, mutant GATA4 contains the functional zinc finger region, which has been shown to interact not only with DNA, but also with other proteins, such as HNF1α[33] and friend of GATA (FOG) cofactors.[53] Indeed, we have shown that although the activation domains of HNF1α are absolutely required for synergy, the GATA activation domains are dispensable for this activity,[33, 34] suggesting that mutant GATA4 maintains the ability to mediate GATA4/HNF1α synergy. Thus, the phenotype attributed to the inactivation of GATA4 in our current model could represent a specific GATA4 function, a masked GATA6 function, and/or a function dependent on GATA4 activation domains. Further, because GATA4 is inducibly inactivated rather than null for GATA4 from the earliest phases of intestinal development, it is possible that gene expression that is dependent on the presence of GATA4 early in development (before administration of tamoxifen) may not be revealed in the current model. Nevertheless, since the same GATA4 model (induction of mutant GATA4) is used throughout our studies, the differential regulation of target genes at diverse developmental time-points continues to support different underlying mechanisms of regulation during development.

Our data show that the induction and maintenance of terminal differentiation by GATA4 and HNF1α is highly dependent on the developmental time-frame under study. Although GATA4 and HNF1α are indispensable for the maintenance of expression of specific genes in the mature intestine[32, 35] consistent with a mechanism of co-regulation, these transcription factors are only partially required for the same genes prior to the final maturation that occurs at weaning, arguing against a co-regulation mechanism and implicating other mechanisms in the development of gut function. Understanding the individual and combined effects of intestinal transcription factors during development will continue to reveal important regulatory pathways essential for intestinal differentiation.

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Chapter 3

GATA4 mediates gene repression in the mature mouse small intestine through interactions with friend of GATA (FOG) cofactors

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ABSTRACT

GATA4, a transcription factor expressed in the proximal small intestine but not in the distal ileum, maintains proximal-distal distinctions by multiple processes involving gene repression, gene activation, and cell fate determination. Friend of GATA (FOG) is an evolutionarily conserved family of cofactors whose members physically associate with GATA factors and mediate GATA-regulated repression in multiple tissues. Using a novel, inducible, intestine-specific *Gata4* knock-in model in mice, in which wild-type GATA4 is specifically inactivated in the small intestine, but a GATA4 mutant that does not bind FOG cofactors (GATA4ki) continues to be expressed, we found that ileal-specific genes were significantly induced in the proximal small intestine ($P<0.01$); in contrast, genes restricted to proximal small intestine and cell lineage markers were unaffected, indicating that GATA4-FOG interactions contribute specifically to the repression function of GATA4 within this organ. *Fog1* mRNA displayed a proximal-distal pattern that parallels that of *Gata4*, and FOG1 protein was co-expressed with GATA4 in intestinal epithelial cells, implicating FOG1 as the likely mediator of GATA4 function in the small intestine. Our data are the first to indicate FOG function and expression in the mammalian small intestine.

INTRODUCTION

The mammalian small intestine is lined by a highly specialized epithelium that displays a wide ranging, yet tightly regulated functional diversity along its cephalo-caudal axis.[1] This functional diversity is linked to a continuous renewal process in which stem cells located at or near the base of crypts produce transit amplifying cells that ultimately differentiate into four principal cell types. Absorptive enterocytes, which constitute the majority of intestinal epithelial cells, enteroendocrine cells, and goblet cells migrate up the villi and are shed into the intestinal lumen every 3 to 5 days, whereas Paneth cells reside at the base of crypts and turn over at a slower rate. Specific absorptive enterocyte genes that encode proteins that mediate absorption of bile salts are localized to the distal ileum, including the apical sodium-dependent bile acid transporter (ASBT)[2] and ileal lipid binding protein (ILBP).[3] Proteins responsible for the terminal digestion and absorption of most nutrients are localized to jejunum and proximal ileum, as exemplified by lactase-phlorizin hydrolase (LPH)[4] and liver fatty acid binding protein (FABP1).[5] Goblet cells are more numerous in distal small intestine,[6] and enteroendocrine subpopulations display a functional diversity characterized by the regional segregation of hormones that activate (e.g. cholecystokinin, CCK) or repress (e.g. peptide YY, PYY) gastrointestinal processes.[7] Maintenance of a dynamic diversity in gene expression and cell fate allocation along the cephalo-caudal axis is necessary for the normal functioning of the small intestine.

Recently, we found that GATA4 is a key regulator of regional gene expression and cell fate allocation in the adult mouse small intestine.[8] GATA4 is a member of a conserved family of transcription factors that contain a pair of zinc fingers that mediate binding to their consensus DNA sequence, WGATAR, in the regulatory region of target genes.[9] In the small intestine of adult rodents and humans, GATA4 is expressed at high levels in proximal regions, but is undetectable in distal ileum.[8, 10] Conditional, inducible inactivation of *Gata4* in the adult mouse jejunum results in a generalized transformation to an ileal-like phenotype that is characterized by an induction of ileal-specific genes, including *Asbt* and *Ilbp*, and attenuation of genes restricted to proximal small intestine, including *Lph* and *Fabp1.* Furthermore, secretory cell fate also becomes ileal-like as indicated by a significant increase in goblet cell number and the abundance of the mRNA for *Math1*, a secretory cell mediator,[11] and a trend toward an increase in *Pyy* mRNA and a decrease in *Cck* mRNA.[8] This novel finding establishes a fundamental plasticity in the adult mammalian small intestine not previously realized, and highlights multiple levels of regulation by GATA4 in this organ involving gene repression, gene activation, and cell fate determination. However, the precise mechanisms by which GATA4 regulates these diverse functions in the mature small intestine are currently unknown.

Friend of GATA (FOG) is an evolutionarily conserved multi-zinc finger cofactor family whose members physically associate with GATA factors, and mediate GATA function in a broad array of tissues and cell types.[12] The GATA-FOG interaction is conserved in *Drosophila* where the FOG homolog, *U-shaped*, physically associates with *pannier*, a GATA homolog,[13] indicating the

fundamental importance of this interaction. Using a split two-hybrid screen, a GATA1 mutant (GATA1ki) with a valine-to-glycine substitution at position 205 in the N-terminal zinc finger was identified that has attenuated binding affinity for FOG cofactors, but normal DNA binding function.[14] *Gata1*$^{ki/ki}$ mice that express GATA1ki in place of GATA1 die during embryogenesis due to anemia caused by disrupted erythroid maturation and megakaryocyte abnormalities.[14] This phenotype is similar to that in *Gata1*$^{-/-}$[15-17] or *Fog1*$^{-/-}$[18] mice, indicating that GATA1-FOG1 interaction is required for hematopoiesis. A FOG1 mutant that restores GATA1-FOG1 interaction rescues this phenotype, providing in vivo evidence that the *Gata1* knock-in mutation specifically disrupts the ability of GATA1 to bind FOG cofactors, and that GATA1ki is otherwise functional.[14, 19] In addition, a *Gata4* knock-in model was designed with the analogous valine-to-glycine substitution at position 217 in GATA4 (GATA4ki) that disrupts the interaction between GATA4 and FOG cofactors.[20, 21] *Gata4*$^{ki/ki}$ mice show an embryonic lethal cardiac phenotype, very similar to that found in *Gata4*$^{-/-}$[22, 23] and *Fog2*$^{-/-}$[24, 25] mice, providing evidence that GATA4-FOG2 interaction is required for cardiogenesis. Furthermore, the *Gata4*^{ki} model has revealed that GATA4-FOG interaction is important in gonadal differentiation[21, 26] and gastric epithelial development.[27]

Using a novel, inducible, intestine-specific *Gata4* knock-in model in mice, in which wild-type GATA4 is specifically inactivated in the small intestine, but a GATA4 mutant that does not bind FOG cofactors (GATA4ki) continues to be expressed, we found that ileal-specific genes were significantly induced in the proximal small intestine ($P<0.01$), but genes restricted to proximal small intestine and cell lineage markers were unaffected, indicating that GATA4-FOG interactions contribute specifically to the repression function of GATA4 within this organ. *Fog1* mRNA displayed a proximal-distal pattern that parallels that of *Gata4*, and FOG1 protein was co-expressed with GATA4 in intestinal epithelial cells, implicating FOG1 as the likely mediator of GATA4 function in the small intestine. These data are the first to indicate FOG function and expression in the mammalian small intestine.

MATERIALS AND METHODS

Mice

Mice were housed under standard conditions in the Animal Research at Children's Hospital (ARCH) facility and provided with food and water ad libitum. Approval was obtained from the Institutional Animal Care and Use Committee. Four *Gata4* alleles were utilized in this study (Figure 1A). The *Gata4*^{flox} allele, which contains loxP sites flanking the translational start site and the region encoding the activation domains of GATA4, expresses wild-type GATA4.[28] Exposure of *Gata4*^{flox} to the recombinase CRE results in exon2 excision (*Gata4*$^{\Delta ex2}$), and the subsequent utilization of an alternative in-frame ATG in exon3 leading to the synthesis of a truncated, transcriptionally inactive form of GATA4 (GATA4Δex2) devoid of its activation domains.[8] The *Gata4*^{ki} allele encodes a mutant GATA4 (GATA4ki) that contains a single amino acid substitution (V217G)

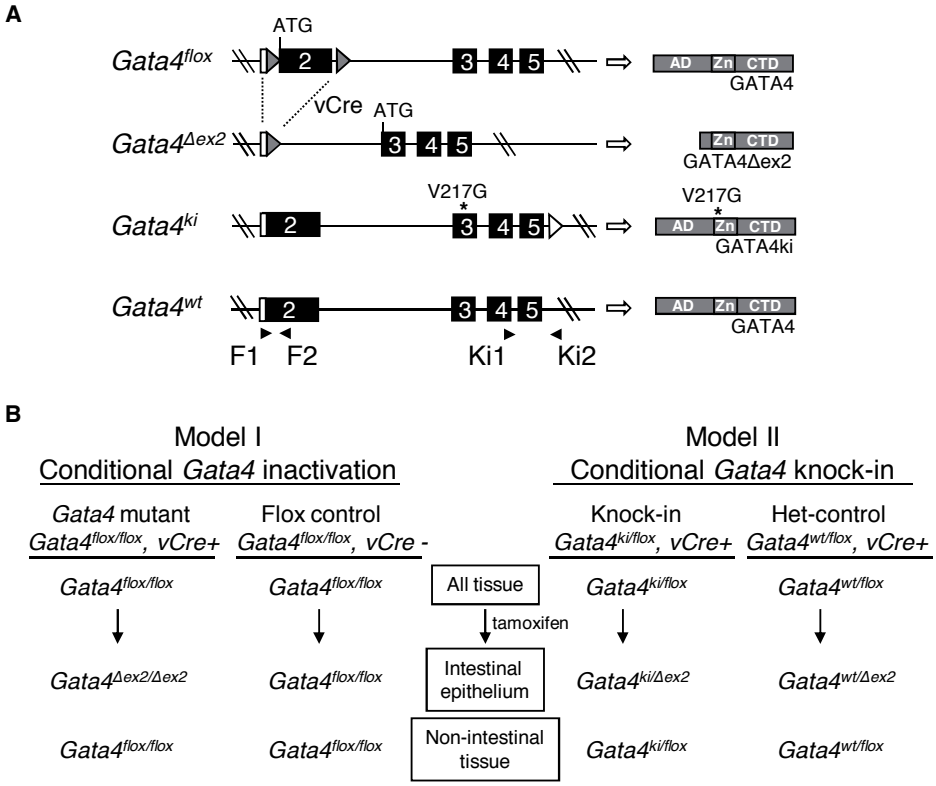

Figure 1. In vivo mouse models. (A) Schematic representation of the different *Gata4* alleles used in this study, and their protein products. Open boxes indicate untranslated region. Filled boxes indicate translated region. Numbers indicate exons. Gray arrowheads indicate loxP sites used for excision of the GATA4 activation domains in the *Gata4flox* allele. Open arrowhead indicates a residual loxP site previously used to remove a neomycin cassette from the *Gata4ki* allele.[20] Gray boxes indicate protein product. AD, activation domain; Zn, zinc fingers; CTD, C-terminal domain. Filled arrowheads indicate PCR primers. (B) Models used in this study showing the *Gata4* alleles in different tissue in test and control mice before and after tamoxifen treatment. vCre indicates the Villin*Cre*ERT2 transgene.

in the N-terminal zinc finger rendering it unable to bind specifically with FOG cofactors.[20] The wild-type allele (*Gata4*^{wt}), was also utilized in the study. Genotyping to distinguish among each of the four *Gata4* alleles was conducted by PCR on DNA extracted from tail biopsies as described[29] using primers specific for exon2 or exon5 of *Gata4* (see Supplementary Figure 1).

Two different conditional, inducible genetic mouse models were used for this study, including a previously validated conditional *Gata4* inactivation model,[8] and a novel conditional *Gata4* knock-in model (Figure 1B). Both models were established in a Villin*Cre*ER^{T2} transgenic background[30] in which CRE-mediated excision of floxed *Gata4* DNA occurs specifically in intestinal and colonic epithelium after tamoxifen treatment.[8] Adult mice (6-8 weeks) were treated with five single intraperitoneal injections of tamoxifen (100µl, 10mg/ml) per day for 5 consecutive days

and sacrificed for tissue collection 14 days after the last injection,[8] unless indicated otherwise. In the *Gata4* inactivation model (Figure 1B, Model I), *Gata4*$^{flox/flox}$, Villin*Cre*ER^{T2}-positive mice were treated with tamoxifen resulting in exon2 deletion (*Gata4*$^{\Delta ex2}$) and subsequent conditional *Gata4* inactivation in the intestinal epithelium.[8] *Gata4*$^{flox/flox}$, Villin*Cre*ER^{T2}-negative mice treated with tamoxifen were used as controls. In the *Gata4* knock-in model (Figure 1B, Model II), *Gata4*$^{ki/flox}$ knock-in mice and *Gata4*$^{wt/flox}$ controls (het-controls) were established in a Villin*Cre*ER^{T2} background. Before tamoxifen treatment, both knock-in mice and het-controls produce wild-type GATA4 from at least one allele in all GATA4 expressing tissue, including the small intestine. After tamoxifen treatment of knock-in mice, GATA4 and GATA4ki are expressed in all tissues that normally express GATA4, except in the intestinal epithelium, where GATA4 from the *Gata4*^{flox} allele is specifically inactivated by CRE-mediated excision, but GATA4ki continues to be expressed from the *Gata4*^{ki} allele. After tamoxifen treatment of het-controls, GATA4 is expressed from both alleles in all tissues that normally express GATA4, except in the intestinal epithelium, where *Gata4*^{flox} is excised by CRE, but GATA4 continues to be expressed from *Gata4*^{wt} allele. This approach creates a conditional, inducible *Gata4* knock-in model.

RNA isolation

RNA was isolated from heart, stomach, and small intestine using the RNeasy kit (Qiagen) as described previously.[8] Intestinal segments (0.5 to 1.0 cm) were obtained from the most proximal region adjacent to the pylorus (segment 1), the 25% mark (segment 2), the geometric center (segment 3), the 75% mark (segment 4), and the most distal region adjacent to the ileocecal junction (segment 5).

Sequencing

To confirm CRE-mediated excision and expression of *Gata4ki,* sequencing analyses were conducted. Complementary DNA (cDNA) was synthesized from jejunal RNA obtained from segment 3, and the region encompassing the knock-in mutation was amplified by PCR. The PCR product was separated on an agarose gel by electrophoresis, extracted using the QIAquick Gel Extraction Kit (Qiagen), re-amplified using the same primers, and purified using ExoSap-IT (USB Corporation). The purified PCR product was sequenced at the Molecular Genetics Core (Children's Hospital Boston) using a nested primer.

RT-PCR

To quantify mRNA abundances, semi-quantitative and real-time RT-PCR were conducted as described previously.[8] Primer pairs (Supplementary Figure 2) were designed using Beacon Design software (Biosoft International) and optimized. Real-time RT-PCR was carried out using an iCycler and iQ SYBR Green Supermix (Bio-Rad). *Gapdh* mRNA abundance was measured for each sample and used to normalize the data. All data were expressed relative to a calibrator as indicated in the figure legends.

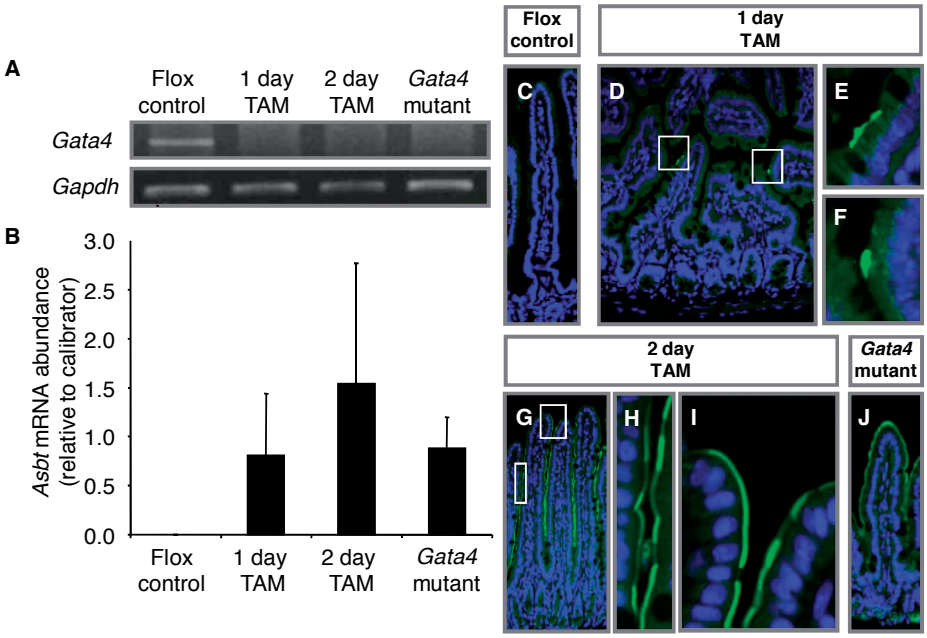

Figure 2. GATA-4 mediated repression of *Asbt expression* occurs in differentiated absorptive enterocytes. *Gata4*$^{flox/flox}$, Villin*Cre*ER^{T2}-positive mice were treated with tamoxifen for 1 day (1 day TAM) or 2 days (2 day TAM) and sacrificed 24 h after the last injection. *Gata4*$^{flox/flox}$ mice, negative (Flox control) or positive (*Gata4* mutant) for the Villin*Cre*ER^{T2} transgene were treated with tamoxifen for 5 days and sacrificed 2 wk later as described.[8] All samples were collected from the geometric center of the small intestine (mid-jejunum). (A) *Gata4* mRNA abundance, determined by semi-quantitative RT-PCR using primers specific for exon 2, reveals' complete CRE-mediated excision after only one treatment of tamoxifen. *Gapdh* was used as a positive control. This finding was replicated on 3 different sets of mice. (B) *Asbt* mRNA is induced within 1 day of a single dose of tamoxifen as determined by real-time RT-PCR (n=3 in each group). RNA from jejunum of a *Gata4* mutant mouse was used as a calibrator. (C-J) ASBT is induced in the microvillus membrane of enterocytes on villi 1 and 2 days after tamoxifen treatment as determined by immunofluorescence (green). Nuclei were counterstained with DAPI (blue).

Immunoblotting

Crude nuclear extracts were isolated from four quarters of intestine (proximal-to-distal: I-IV) and Western analysis was conducted as described previously[10] using 100 μg of extract. The membranes were blocked for 1 h at room temperature in 5% nonfat dried milk in PBS and incubated with goat anti-FOG1 (1:1500, Santa Cruz) with or without FOG1 blocking peptide (1:1500, Santa Cruz) for 1 h. Membranes were stripped and re-probed using mouse anti-β-actin (1:4000, Santa Cruz). Horseradish peroxidase-linked secondary antibodies and chemiluminescence solution (Pierce West Femto Kit) were used to visualize FOG1 or β-actin signals.

In situ hybridization

RNA probes were prepared by in vitro transcription of a partial cDNA insert from the *Fog1* library plasmid M10 subcloned into pBluescript KS (Stratagene)[31] using digoxigenin-UTP (Roche Molecular Biochemicals), and T3 (antisense) or T7 (sense) polymerase as described.[32] In situ hybridization assays were conducted as described previously.[25]

Immunohistochemistry

Intestinal segments were fixed in ice-cold 4% paraformaldehyde in PBS for 4 h, dehydrated overnight as described,[8] embedded in paraffin, and sectioned (5 μm) in the Department of Pathology at Children's Hospital Boston. After deparaffinization and antigen retrieval,[8, 29] the sections were incubated with the primary antibody for 1 h at 37°C, rinsed, and then incubated with the secondary antibody for 1 h at 37°C. For immunofluorescence, sections were incubated in a solution containing 4',6-diamino-2-phenylindol dihydrochloride (DAPI, 2 μg/ml, Molecular Probes) in PBS for 15 min at room temperature, washed in PBS, and mounted in Mowiol mounting medium (Calbiochem). For immunohistochemistry, biotinylated secondary antibodies were linked to avidin-horseradish peroxidase or avidin-alkaline phosphatase conjugates (Vector Labs), and visualized using 3,3'-diamino benzidine (DAB) for 2-5 min or 4-nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3indolyl-phosphate (BCIP) for 20-90 min, respectively. For selected sections, the tissue was lightly counterstained with methyl green.

The primary antibodies included rabbit anti-ASBT (1:500, kind gift of Dr. P.A. Dawson, Wake Forest School of Medicine), goat anti-FOG1 (1:200, Santa Cruz), mouse anti-GATA4 (1:400, Santa Cruz), goat anti-GATA4 (1:400, Santa Cruz), rabbit anti-chromogranin A (1:1000, Immunostar), rabbit anti-lysozyme (1:200, Zymed), and rabbit anti-Ki67 (1:100, Zymed). The secondary antibodies included Alexa fluor 488 anti-rabbit IgG (1:500, Invitrogen), biotinylated anti-goat IgG (1:500, Vector Labs), and biotinylated anti-rabbit IgG (1:500, Vector Labs).

Statistical analyses

A total of 16 knock-in and 10 het-control mice were analyzed. Due to unequal variances for certain data sets, the median and individual data points are presented, and statistically significant differences, indicated by a P-value of less than 0.05, were determined by the nonparametric Mann-Whitney U-test. For data sets in which statistical analysis was not performed, the mean and standard deviation are indicated.

RESULTS

GATA4-mediated repression of *Asbt* gene expression occurs in differentiated absorptive enterocytes

As previously shown,[8] conditional, inducible *Gata4* inactivation by excision of the activation domains of GATA4 (synthesis of GATA4Δex2), results in an induction in jejunum of absorptive enterocyte genes normally restricted to distal ileum, including *Asbt* and *Ilbp.* Since GATA4 is expressed in both crypt and villus epithelial cells,[8, 10, 33, 34] it is uncertain whether GATA4 represses ileal-specific genes in proximal small intestine by a process that occurs in differentiated absorptive enterocytes on villi, and/or is determined early in the differentiation program in crypt progenitor cells. To localize the site of action for GATA4-mediated repression of intestinal genes, the induction of *Asbt* expression during the initial phases of *Gata4* inactivation was characterized. *Gata4*$^{flox/flox}$ mice positive for the Villin*Cre*ER^{T2} transgene were treated for 1 day or 2 days with tamoxifen, and sacrificed 24 h after the last injection. In mid-jejunum, CRE-mediated excision of *Gata4* was complete after only one injection of tamoxifen (Figure 2A), and *Asbt* mRNA was induced (Figure 2B). Because of the 3-day crypt-to-villus tip cell migration time in mice,[1] we hypothesized that if *Asbt* was induced directly in differentiated absorptive enterocytes, then ASBT would first be detected in a random, patchy pattern throughout the villi. Conversely, if *Asbt* was induced by a process that originated in crypts, then it would first appear in lower villi and migrate up the villi over time. ASBT, not normally present in mid-jejunum (Figure 2C),[35, 36] was first detected in the microvillus membrane of single cells scattered throughout the villi after 1 day of *Gata4* inactivation (Figure 2D-F), and was increasingly expressed in a patchy pattern along the length of the villi extending to the villus tip after 2 days of *Gata4* inactivation (Figure 2G-I). ASBT induction in the mid-jejunum of *Gata4* mutant mice is shown as a control (Figure 2J). Although the design and outcome of this experiment cannot rule out the possibility that a component of GATA4-mediated *Asbt* repression in the jejunum is directed by GATA4 in crypt cells, these data are consistent with the hypothesis that GATA4 mediates *Asbt* repression in differentiated absorptive enterocytes on villi.

Inducible Villin*Cre*ER^{T2}-mediated recombination of the *Gata4*^{flox} allele in *Gata4*$^{ki/flox}$ mice results in an intestine-specific *Gata4* knock-in model

FOG cofactors mediate GATA-regulated repression of specific genes in multiple non-intestinal systems,[14, 31, 37-42] but their function and expression in the small intestine is unknown. To test the hypothesis that FOG cofactors mediate the GATA4-regulated repression of ileal-specific absorptive enterocyte genes in the proximal small intestine, we established *Gata4*$^{ki/flox}$, Villin*Cre*ER^{T2}-positive (knock-in) mice, and *Gata4*$^{wt/flox}$, Villin*Cre*ER^{T2}-positive heterozygous controls (het-controls) (Figure 1). All genotypes were confirmed by PCR using primer pairs that distinguish the *Gata4*^{ki} from the *Gata4*^{flox} or *Gata4*^{wt} alleles, as well as the *Gata4*^{flox} from the *Gata4*^{ki} or *Gata4*^{wt} alleles (Supplementary Figure 3A).

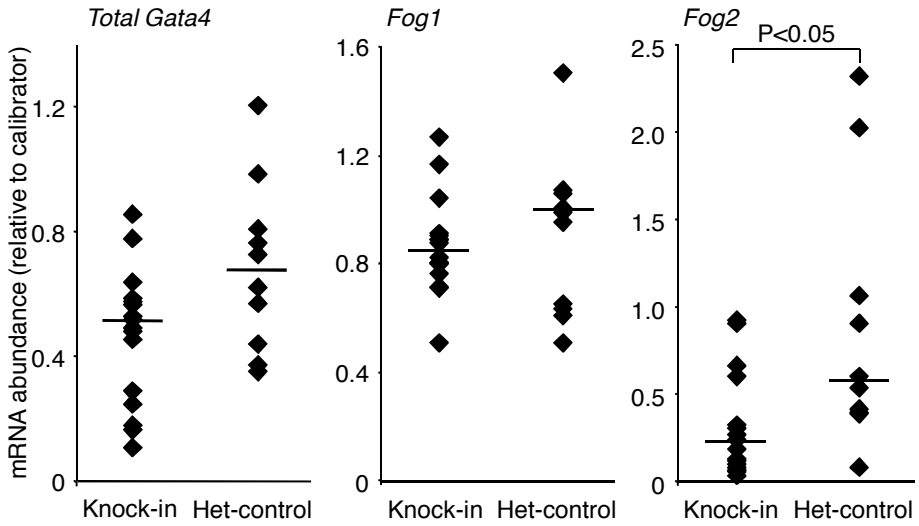

Figure 3. *Fog2* mRNA abundance is lower in *Gata4* knock-in mice as compared to het-controls. *Gata4*, *Fog1*, and *Fog2* mRNA abundances were determined by real-time RT-PCR of RNA isolated from the mid-jejunum of *Gata4* knock-in mice and het-controls. Filled diamonds represent single data points from individual mice. Bars indicate medians. RNA from jejunum of a het-control mouse was used as a calibrator for *Gata4* and *Fog1*, whereas pooled RNA from ileum of 3 wild-type mice was used as a calibrator for *Fog2*.

To validate intestine-specific CRE-mediated excision of the $Gata4^{flox}$ alleles, and specific allelic expression from the $Gata4^{ki}$ allele in the knock-in mice, and from the $Gata4^{wt}$ allele in the het-controls, RT-PCR and cDNA sequencing was conducted on RNA samples from mid-jejunum. The $Gata4^{flox}$ allele, common to both knock-in and het-control mice, was excised in jejunum but not heart after tamoxifen treatment (Supplementary Figure 3B), verifying intestine-specific CRE-mediated excision. To distinguish expression from the $Gata4^{ki}$ vs. $Gata4^{wt}$ alleles, the region encompassing the V217G knock-in mutation was amplified by RT-PCR using the Ki3 and Ki4 primers (Supplementary Figure 3C). Because Ki3 hybridizes to exon2 sequence, and because this exon is excised from the $Gata4^{flox}$ allele after tamoxifen treatment producing the recombined $Gata4^{\Delta ex2}$ allele, the *Gata4Δex2* cDNA is not amplified. Using a nested primer (Ki5), the knock-in or wild-type cDNA was then confirmed by sequencing (Supplementary Figure 3C). For all mice in this study, there was no evidence of band ambiguity at the knock-in site for *Gata4* knock-in mice, verifying that CRE-mediated excision was complete.

The expression of *Gata4*, *Fog1*, and *Fog2* in the knock-in mice and het-controls was determined (Figure 3). Because the $Gata4^{ki}$, $Gata4^{flox}$, and $Gata4^{wt}$ alleles are all under the control of the endogenous *Gata4* promoter, we quantified total *Gata4* mRNA abundance as the most sensitive indicator of a possible knock-in effect on *Gata4* expression. Total *Gata4* and *Fog1* mRNA abundances were not significantly different between the two groups, but *Fog2* mRNA was significantly

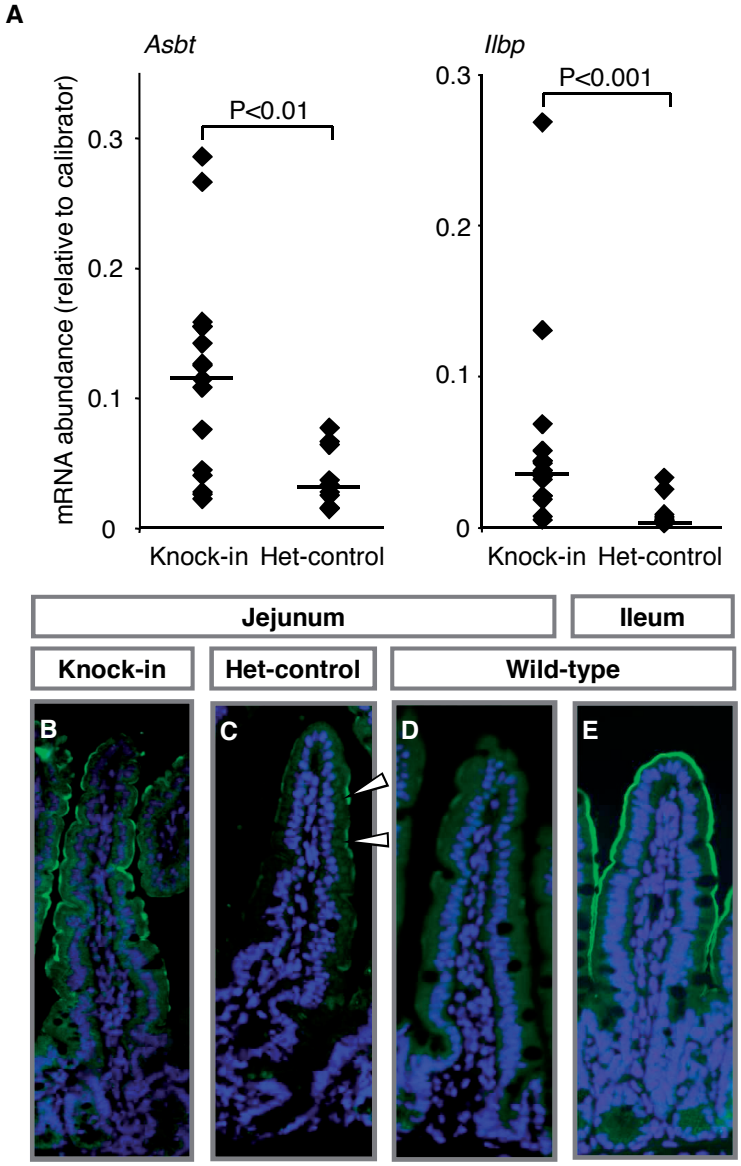

Figure 4. *Asbt* and *Ilbp* expression is induced in the *Gata4* knock-in mice as compared to het-controls. (A) *Asbt* and *Ilbp* mRNA abundances were determined by real-time RT-PCR on RNA isolated from the mid-jejunum of *Gata4* knock-in mice and het-controls. Data are represented as indicated in the legend for Figure 3. Pooled RNA from ileum of 3 wild-type mice was used as a calibrator. (B-E) ASBT was identified by immunofluorescence (green) for representative samples of knock-in jejunum (B), het-control jejunum (C), wild-type jejunum (D) and wild-type ileum (E). Nuclei were counterstained with DAPI (blue). White arrowhead indicates positive ASBT immunofluorescence (C).

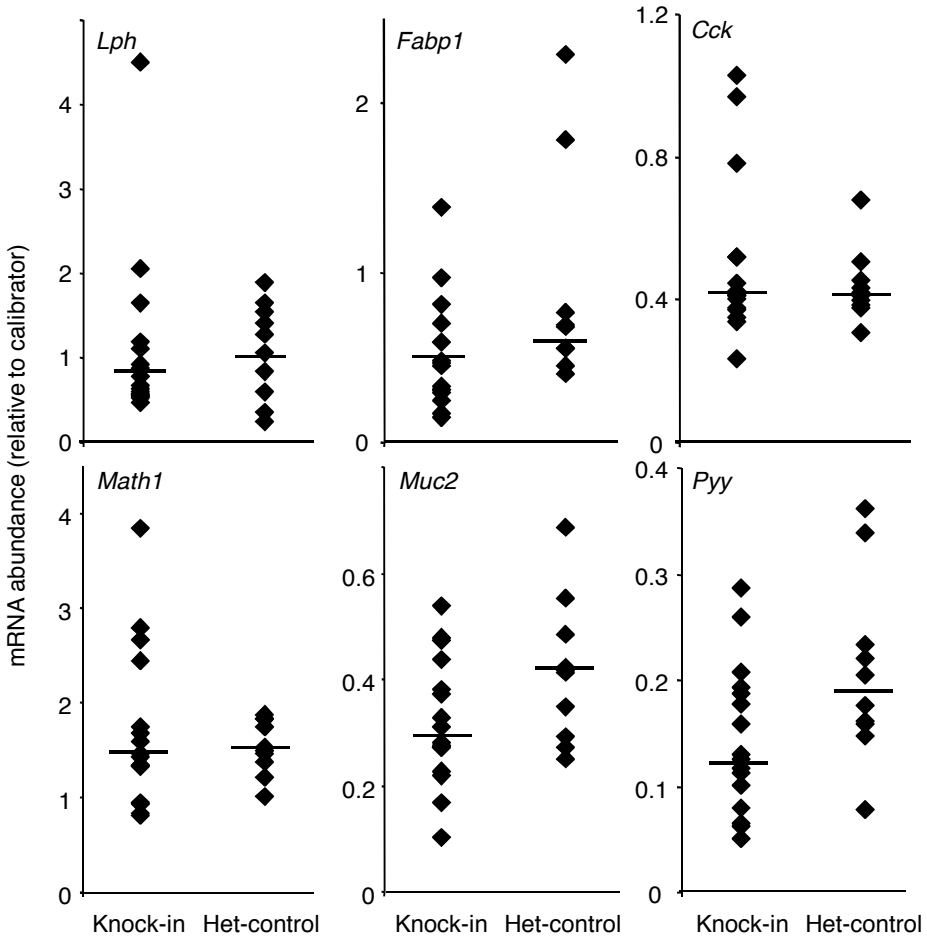

Figure 5. GATA4-mediated activation pathway in absorptive enterocytes and cell lineage markers were not significantly different between *Gata4* knock-in mice and het-controls. The mRNAs for the GATA4-regulated activation pathway in absorptive enterocytes (*Lph* and *Fabp1)* and for cell lineage (*Math1, Muc2, Cck, Pyy*) were determined by real-time RT-PCR on RNA isolated from the mid-jejunum of *Gata4* knock-in mice and het-controls. Data are represented as indicated in the legend for Figure 3. Pooled RNA from jejunum of 3 wild-type mice was used as a calibrator for *Lph, Fabp1, Math1* and *Cck*, whereas pooled RNA from ileum of 3 wild-type mice was used as a calibrator for *Muc2* and *Pyy*.

lower (60%, P<0.05) in the knock-in mice as compared to the het-controls. Noteworthy, the cycle threshold (C_T) for *Fog1* was ~9 cycles lower as compared to *Fog2* indicating that *Fog1* mRNA abundance is 1000-fold higher than that of *Fog2*. These data suggest that although *Fog2* mRNA is expressed at low levels, its abundance is dependent on GATA4-FOG interactions.

Asbt and *Ilbp* expression is induced in *Gata4* knock-in mice

To test the hypothesis that FOG cofactors specifically mediate GATA4-regulated repression of ileal-specific absorptive enterocyte genes in proximal small intestine, the jejunal expression of absorptive enterocyte target genes and lineage markers were compared between *Gata4* knock-in mice and het-controls. As shown in Figure 4, the mRNA abundances of *Asbt* and *Ilbp* (Figure 4A) in jejunum of the knock-in mice were induced 4-fold ($P<0.01$) and 14-fold ($P<0.001$), respectively, compared to the het-controls, consistent with a requirement of GATA4-FOG interactions for repression of ileal-specific genes in the jejunum. Noteworthy, *Asbt* and *Ilbp* mRNAs, which are known to be undetectable in wild-type mid-jejunum,[8, 35, 36, 43] were induced in the jejunum of the het-control mice, indicating either a heterozygous effect, or a dominant negative effect of GATA4Δex2 on wild-type GATA4, suggesting that repression of *Asbt* and *Ilbp* by GATA4 is dose-dependent in vivo. ASBT was highly induced on the microvillus membrane of villus enterocytes in the *Gata4* knock-in mice (Figure 4B), and was detected in isolated cells in the het-controls (Figure 4C), generally consistent with *Asbt* mRNA abundances in the two groups. Undetectable expression in wild-type jejunum (Figure 4D) and endogenous expression in wild-type ileum (Figure 4E) are shown for reference.

The mRNA abundances for the GATA4-mediated activation pathway in absorptive enterocytes (*Lph* and *Fabp1*), and for cell lineage markers (*Math1*, *Muc2, Cck* and *Pyy*) did not reveal any statistically significant differences (Figure 5), suggesting that GATA4-FOG interaction is not required for GATA4-mediated activation of absorptive enterocyte genes, or maintenance of jejunal cell lineage distribution patterns.

FOG1 demonstrates a decreasing proximal-to-distal expression pattern in adult mouse small intestine

To begin to elucidate which FOG cofactor may be responsible for mediating GATA4 function, the expression patterns of *Fog1* and *Fog2* were determined in the adult mouse small intestine. *Fog1* mRNA abundance demonstrated a decreasing proximal-to-distal pattern (Figure 6A, upper) similar to that of *Gata4*.[8, 10] The abundance of *Fog1* mRNA in proximal duodenum (segment 1) was similar to that in stomach, but in distal ileum (segment 5) was ~10% of that in stomach, and similar to the low level of expression in heart. *Fog2* mRNA was low proximally and increased distally (Figure 6A, lower), a pattern reciprocal to that of *Gata4* and *Fog1*. Intestinal *Fog2* mRNA abundance was similar or less than that in stomach, and ranged from 7 to 20% of that in heart.

To determine whether FOG proteins mimic their mRNA distributions, Western blot analyses were carried out. Since *Fog2* mRNA abundance was low in the small intestine (~1000-fold less than *Fog1*), and FOG2 protein could not be detected by immunohistochemistry (data not shown), only protein patterns for FOG1 were analyzed. As shown in Figure 6B, a 160 kDa band corresponding to FOG1[19] demonstrated a declining proximal-to-distal pattern similar to that of its mRNA profile. The 160 kDa band, as well as bands with faster mobilities, were specifically blocked by an epitope-specific polypeptide, verifying specific antigen detection. Taken together, these data

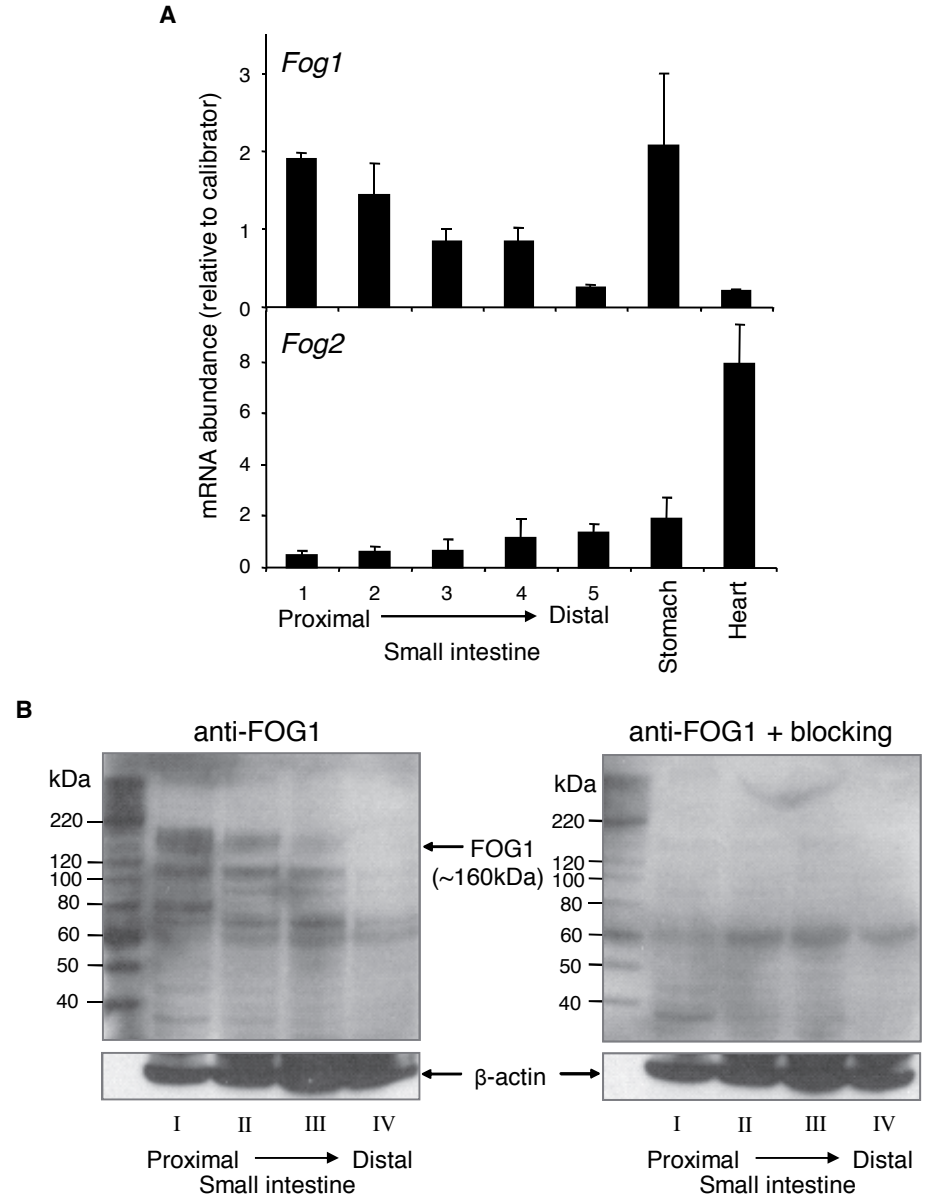

Figure 6. FOG1 demonstrates a decreasing proximal-to-distal expression pattern in adult mouse small intestine. (A) *Fog1* (upper) and *Fog2* (lower) mRNAs were determined by real-time RT-PCR on RNA obtained from segments 1-5 from wild-type mouse small intestine, stomach, and heart (n=3 in each group). RNA from jejunum of a wild-type mouse was used as a calibrator. (B) FOG1 was identified by Western blot analysis on crude nuclear extracts isolated from quarters I-IV (proximal-to-distal) of wild-type mouse small intestine using a goat anti-FOG1 antibody (Santa Cruz) and an anti-goat IgG secondary antibody (Santa Cruz), without (left) or with (right) an epitope-specific blocking peptide. Blots were re-probed with an anti-β-actin antibody.

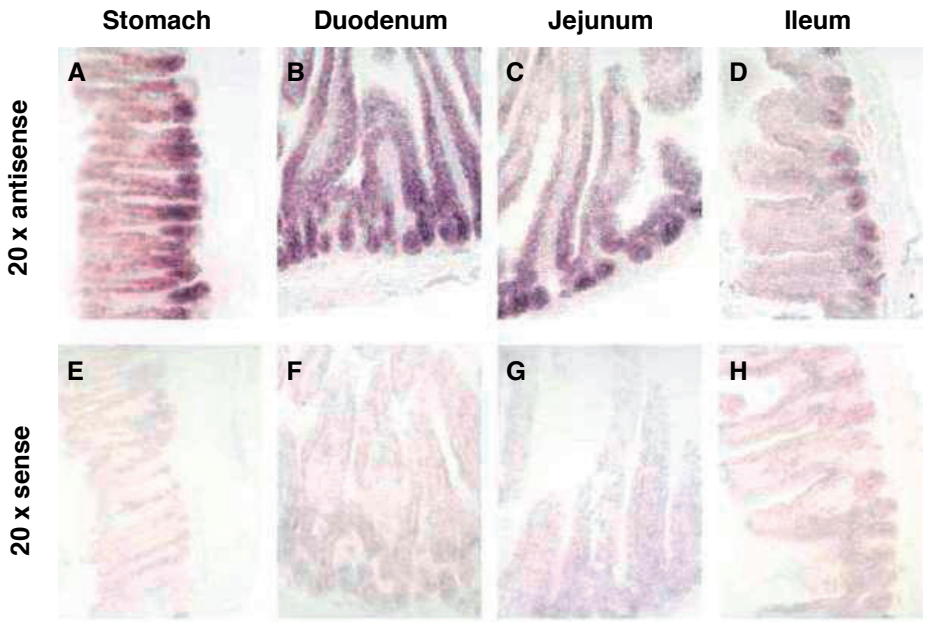

Figure 7. *Fog1* mRNA is expressed in a distinct crypt-villus and proximal-distal pattern in the adult mouse small intestine. In situ hybridization assays were conducted using antisense (A-D) and sense (E-H) *Fog1* probes, and images at 20x magnification are shown. Analysis of stomach (A, E), duodenum (B, F), jejunum (C, G), and ileum (D, H) reveal a generally decreasing proximal-to-distal signal intensity with the greatest intensity localized to the base of the gastric gland (stomach) and in crypts (small intestine).

demonstrate that FOG1 has a declining proximal-to-distal expression pattern in the adult small intestine that is similar to that of GATA4.

To define *Fog1* mRNA expression at the cellular level, in situ hybridization assays were carried out on stomach and small intestine of wild-type mice (Figure 7). *Fog1* mRNA was highly expressed in the stomach with the strongest signal localized to the base of the gastric gland (Figure 7A). *Fog1* mRNA was detected throughout the small intestine, but revealed a clear proximal-to-distal decrease in signal intensity and cellular localization (Figure 7B-D), corroborating the quantitative cephalo-caudal decrease in *Fog1* mRNA and protein. *Fog1* mRNA was detected in crypts and villi in duodenum, crypts and lower villi in jejunum, and only in crypts in ileum. A sense control was used to indicate background (Figure 7E-H).

FOG1 is co-expressed with GATA4 in absorptive enterocytes on villi, and in lysozyme-positive and proliferating cells in the crypt of the adult mouse small intestine

To delineate the cellular expression patterns of FOG1 in the small intestine, and its co-localization with GATA4, immunostaining for FOG1 and GATA4, along with lineage-specific markers, was conducted on proximal jejunum of adult mouse small intestine. We had previously demonstrated

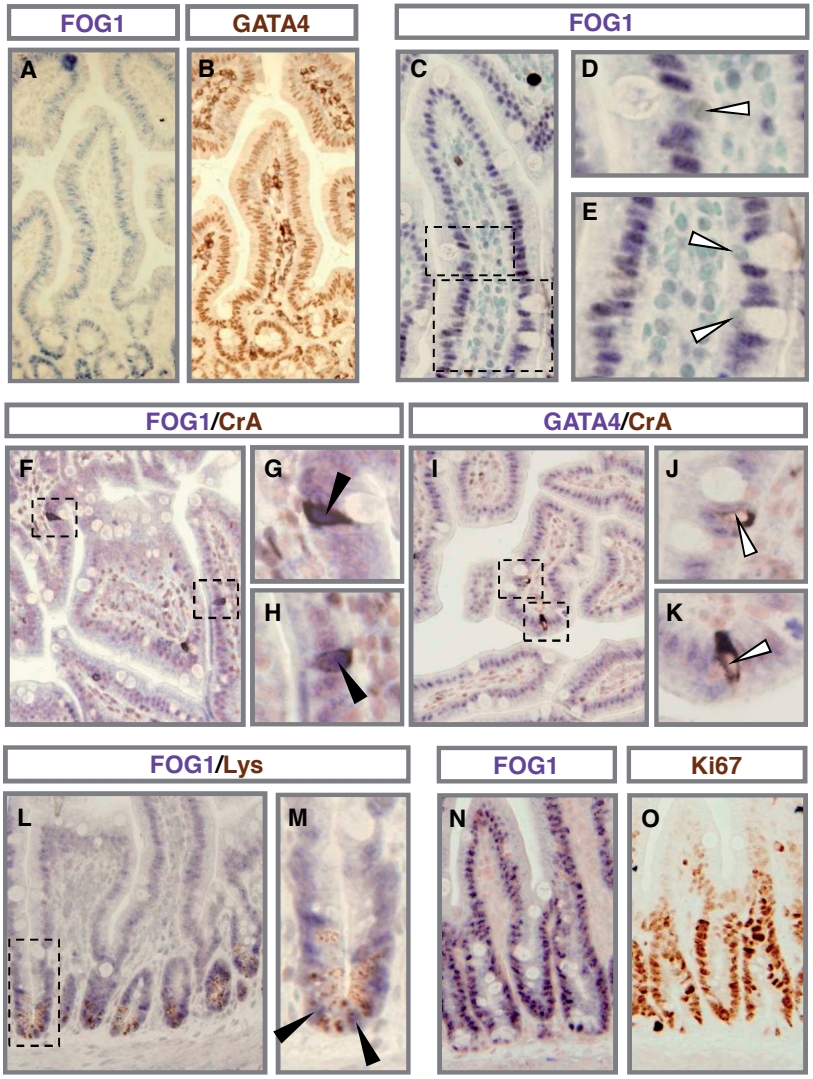

Figure 8. FOG1 is co-expressed with GATA4 in absorptive enterocytes on villi and in lysozyme-positive and proliferating crypt cells. Jejunal sections obtained from adult wild-type mice were used for all immunostaining. Immunostaining for FOG1 (A) or GATA4 (B) on serial sections demonstrate that FOG1 and GATA4 are co-expressed. Nuclei (counterstained with methyl green) of goblet cells do not stain for FOG1 (C-E). Immunostaining with antibodies for FOG1 (F-H) or GATA4 (I-K), co-stained with chromogranin-A (F-K), show that FOG1 is expressed in the nuclei of chromogranin-A positive enteroendocrine cells, but GATA4 is not. Immunostaining with antibodies for FOG1 and lysozyme (L,M) demonstrate that FOG1 is expressed in lysozyme-positive cells. Serial sections stained for FOG1 (N) or Ki67 (O) show that FOG1 is expressed in Ki67-positive proliferating cells in crypts. Open arrowheads indicate an absence of FOG1 or GATA4 staining; filled arrowheads indicate FOG1-positive immunostaining.

that GATA4 is expressed in absorptive enterocytes on villi, and in proliferating and lysozyme-positive crypt cells, but is not expressed in goblet or enteroendocrine cells.[8] Using an alkaline phosphatase substrate detection approach, FOG1 was identified in the nuclei of epithelial cells throughout the crypts and villi (Figure 8A), and serial section immunostaining revealed co-localization with GATA4 (Figure 8B) in both compartments. FOG1 immunostaining could not be detected in the nuclei of goblet cells (Figure 8C-E), as with GATA4,[8] but FOG1 immunostaining was detected in the nuclei of chromograninA-positive enteroendocrine cells (Figure 8F-H), contrasting with the absence of GATA4 immunostaining in this lineage (Figure 8I-K).[8] FOG1 immunostaining was also detected in lysozyme-positive (Figure 8L, M) and proliferating (Figure 8N, O) crypt cells, and was thus co-localized with GATA4 in this compartment.[8] Taken together, these data show that FOG1 and GATA4 are co-localized in absorptive enterocytes on villi, and in lysozyme-positive and proliferating crypt cells of the adult mouse small intestine, demonstrating a topographic basis for possible interactions. These data also reveal divergent expression in chromograninA-positive enteroendocrine cells, suggesting that FOG1 has functions in this lineage that are independent of GATA4.

DISCUSSION

GATA4, a transcription factor expressed in the proximal small intestine but not in the distal ileum, maintains proximal-distal distinctions in the mature small intestine by multiple processes involving gene repression, gene activation, and cell fate determination.[8] Since interactions between GATA factors and FOG cofactors result in the repression of GATA-mediated transcriptional activation of hematopoietic and cardiac target promoters,[14, 31, 37-42] we tested the hypothesis that FOG cofactors are necessary for GATA4-mediated repression of ileal-specific absorptive enterocyte genes in the small intestine in vivo. We engineered a novel, inducible, intestine-specific *Gata4* knock-in model (Figure 1), in which wild-type GATA4 is specifically inactivated in the intestine, but a GATA4 mutant that does not bind FOG cofactors (GATA4ki) continues to be expressed. We found that ileal-specific genes are induced in the proximal small intestine (Figure 4), whereas genes restricted to proximal small intestine and cell lineage markers are unaffected (Figure 5), indicating that GATA4-FOG interactions contribute specifically to the repression function of GATA4 within this organ. Furthermore, co-expression of GATA4 and FOG1 in the nuclei of absorptive enterocytes on villi, and throughout the crypt epithelium (Figure 8), suggests that FOG1 mediates GATA4-regulated repression of intestinal genes, although a role for FOG2 in this process cannot be discounted. These findings provide the first indication of FOG function and expression in the mammalian small intestine.

Repression of specific genes by GATA factors is well documented,[14, 31, 37, 39, 40, 42] and generally occurs by recruitment of FOG cofactors to target gene promoters, which, in turn, mediates recruitment of the nucleosome remodelling and histone deacetylase (NuRD) complex leading

to the deacetylation of local histones and gene silencing.[38, 41] Our data show that, GATA4 and FOG cofactors, specifically FOG1, are co-expressed in differentiated absorptive enterocytes on villi (Figure 8), and are consistent with the hypothesis that GATA4 mediates *Asbt* repression in differentiated absorptive enterocytes on villi rather than by an upstream process dictated earlier in the differentiation process in crypt progenitor cells (Figure 2). These findings have led us to hypothesize that GATA4 mediates *Asbt* repression by binding directly to the *Asbt* promoter. While it is possible that an indirect pathway within absorptive enterocytes, such as repression of another activator, or activation of a repressor, could mediate *Asbt* repression, we have previously shown that the mRNAs for known activators of *Asbt*, including hepatocyte nuclear factor-alpha (HNF1α),[44] liver receptor homolog-1 (LRH1),[45] and c-FOS,[46] are not decreased in our conditional *Gata4* inactivation model.[8] Thus, we hypothesize that GATA4-regulated repression of specific intestinal genes is mediated by promoter-dependent recruitment of GATA4-FOG complexes that may, in turn, promote histone deacetylation and gene silencing by recruitment of the NuRD complex. This model assumes that GATA4-FOG repression in the proximal small intestine hierarchically overrides activation by HNF1α, LRH1, c-FOS, or any other as yet unknown activator of *Asbt* gene expression.

Our data show that while the gene repression pathway is dependent on GATA4-FOG interactions (Figure 4), the gene activation pathway is independent of this interaction (Figure 5). We and others have previously shown using in vitro and cell culture models that GATA4 physically associates with HNF1α and synergistically activates the *Lph* and *Fabp1* promoters[10, 33] through an evolutionarily conserved mechanism.[47] Furthermore, both genes are strongly attenuated in intestine of mice in which *Gata4* is conditionally inactivated,[8, 48] or *Hnf1α* is knocked out.[29, 48] These findings support our original hypothesis that the overlapping expression of GATA4 and HNF1α in the intestinal epithelium, combined with specific promoter signatures in target genes, results in the activation of a specific subset of genes in proximal small intestine.[10, 47, 49] It is intriguing to speculate that the promoter configurations in genes activated by GATA4-HNF1α cooperativity specifically exclude the recruitment of GATA4-FOG complexes.

We had previously shown that conditional inactivation of *Gata4* results in a significant increase in goblet cell number in the jejunum, and an increase in the expression of *Math1*,[8] a mediator of secretory lineages in the intestine,[11] consistent with a jejunum-to-ileum transformation in cell fate allocation. We also previously found in the jejunum a trend toward an increase in the mRNA abundance of *Muc2*, a goblet cell marker,[6] and a trend toward a decrease in the mRNA for *Cck* and increase in that for *Pyy*, both of which are enteroendocrine cell markers that normally show an increasing and decreasing proximal-distal gradient,[7] respectively. In the present study, we found no evidence that FOG cofactors are required for GATA4-regulated cell fate allocation in the intestine (Figure 5). However, it should be noted that jejuno-ileal differences in cell lineage composition are subtle, and that these differences may be obscured by a gene dosage effect due to the heterozygous nature of the controls, and thus a role for GATA4-FOG interactions in these processes cannot be ruled out.

Both *Fog1* and *Fog2* mRNAs are expressed in the adult mouse small intestine, but *Fog1* is more abundant (~1000-fold), and demonstrates a quantitative pattern that is similar to that of *Gata4* (Figure 6). In humans, *Fog1* mRNA is expressed in the proximal small intestine,[50] whereas *Fog2* mRNA cannot be detected,[51] supporting the relative differential abundances of *Fog1* and *Fog2* mRNAs in the mouse small intestine. FOG1 and GATA4 are co-expressed in absorptive enterocytes on villi, and throughout the proliferating and lysozyme-positive Paneth cells in crypts (Figure 8), suggesting that FOG1 mediates GATA4 function. While FOG1 is generally associated with GATA1, and FOG2 with GATA4, FOG1 was shown to be necessary for heart development[32] presumably reflecting cooperation with the GATA4/5/6 subfamily. FOG1 is also expressed in the chromograninA-positive enteroendocrine cells while GATA4 is not expressed in these cells,[8] suggesting that FOG1 has functions in these cells that are independent of GATA4, possibly through interaction with GATA6, which is expressed in this lineage.[34] *Fog2* mRNA is detectible in the adult mouse small intestine (Figure 6A), and is decreased in the *Gata4* knock-in mice as compared to the het-controls (Figure 3), suggesting that its levels are regulated by GATA4-FOG interactions. Although our data support a role for FOG1 in mediating GATA4 function, we cannot rule out the possibility that FOG2, or both FOG1 and FOG2, are required for GATA4 function in the intestine. Correlation of our knock-in results with those of conditional *Fog1* and *Fog2* single and double knockout will allow a precise determination of FOG1 and FOG2 requirements for GATA4 function in this organ.

The demonstration of a GATA4-FOG requirement for gene expression in the small intestine adds to the growing list of tissues and cell types that require GATA-FOG interactions for normal function. In addition to the well documented role of GATA1-FOG1 interactions in hematopoiesis,[12] GATA4-FOG interaction is required for coronary vasculature initiation, cardiac morphogenesis and valve formation during cardiogenesis,[20] differentiation of precursors into XY-specific Sertoli cells during gonad development,[21] and epithelial-mesenchymal signaling during stomach development.[27] While these studies all highlight the critical roles of GATA4-FOG interactions for specific aspects of embryonic development, our study, due to the inducible nature of our model, is the first to demonstrate a role for GATA4-FOG interactions in adult intestine. Taken together, these studies underscore the diverse nature of GATA4-FOG functions, including morphogenesis, cell fate determination, and maintenance of terminal differentiation.

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SUPPLEMENTAL FILES

Genotyping/sequencing primers:

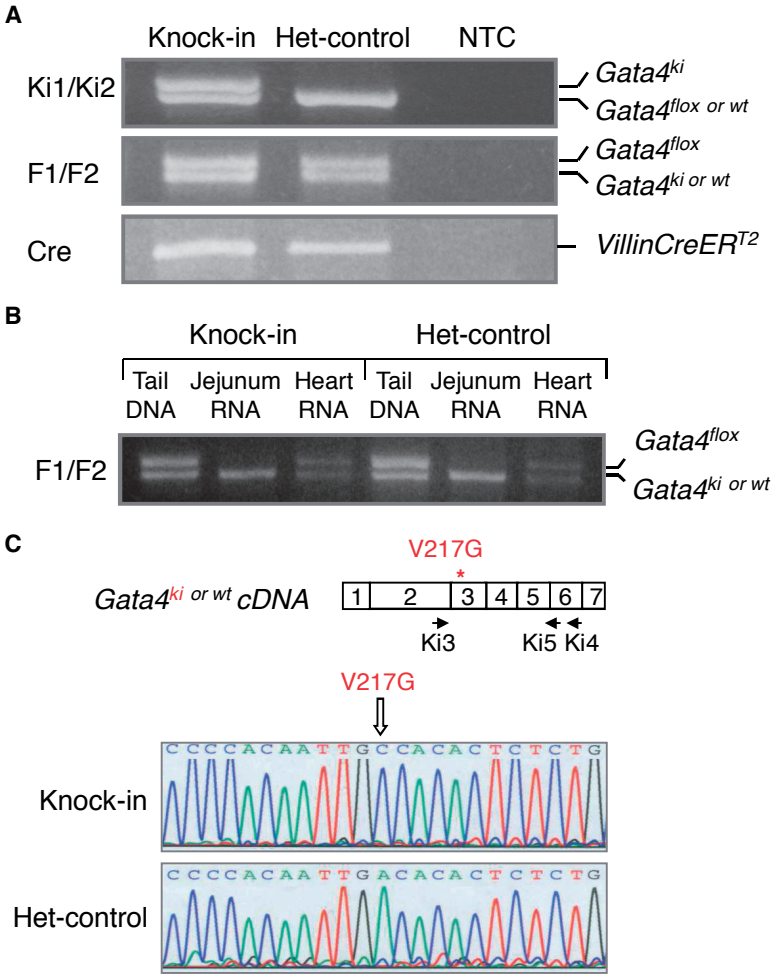
Cre:	F: 5'-CGTATAGCCGAAATTGCCAG-3'
	R: 5'-CAAAACAGGTAGTTATTCGG-3'
Gata4:	
F1:	F: 5'-GGTGGTTTCATTTGCTGTGTGGAAG-3'
F2:	R: 5'-AATCGTGCGGGAGGGCGGACTCTATTC-3'
Ki1:	F: 5'-GGGTGAGCCTGTATGTAATGCCTGCG-3'
Ki2:	R: 5'-GATGACACTGCTTCTGTGGGGTCTTGAG-3'
Ki3:	F: 5'-AACCCTGGAAGACACC-3'
Ki4:	R: 5'-CATTGCTGGAGTTACCG-3'
Ki5:	R: 5'-CTGGAGGCACCACTGG-3'

Supplementary Figure 1. Genotyping and sequencing primers.

Real time RT-PCR primers:

Gata4 :	F: 5'-GAGCCTGCCAAGCCAAGC-3' R: 5'-CTCCCGTCTATCACCTTTGTCC-3'
Fog1:	F: 5'-AGACCAGAGCCTTATCCC-3' R: 5'-GCGTCATCCTTCCTGTAG-3'
Fog2:	F: 5'-CTCTCATTTGCTTGCTCATCTCC-3' R: 5'-GCGGTGTCTGCGGTTCC-3'
Asbt:	F: 5'-TTGCCTCTTCGTCTACACC-3' R: 5'-CCAAAGGAAACAGGAATAACAAG-3'
Ilbp:	F: 5'-TGGCAAAGAATGTGAAATG-3' R: 5'-CTCCGAAGTCTGGTGATAG-3'
Lph:	F: 5'-CAGCGATGCCCACAGGAAAG-3' R: 5'-ACGGAGCCCTTGACGAGAG-3'
Fabp1:	F: 5'-GGTGTCAGAAATCGTGC-3' R: 5'-CAATGTCGCCCAATGTC-3'
Math1:	F: 5'-CCAGCAAACAGGTGAATG-3' R: 5'-TTCTTGTCGTTGTTGAAGG-3'
Muc2:	F: 5'-AACTACCACTGTGATGCCAATG-3' R: 5'-ACAATGTTGATGCCAGACTCG-3'
Cck:	F: 5'-AGGAAACAACCACACATACG-3' R: 5'-AGCATAGCAACATTAGGTCTG-3'
Pyy:	F: 5'-CGACAGCGACAGCGAGAAC-3' R: 5'-AGGGACAGGGAAATGAACACAC-3'
Gapdh:	F: 5'-GCCTTCCGTGTTCCTACCC-3' R: 5'-TGCCTGCTTCACCACCTTC-3'

Supplementary Figure 2. Real time RT-PCR primer sequences.



Supplementary Figure 3. Verification of a conditional, inducible *Gata4* knock-in model. (A) The presence of specific *Gata4* alleles was confirmed by PCR using primer pairs that distinguish the *Gata4*^{ki} from the *Gata4*^{flox} or *Gata4*^{wt} alleles (Ki1/Ki2), as well as the *Gata4*^{flox} from the *Gata4*^{ki} or *Gata4*^{wt} alleles (F1/F2) (see Figure 1 for primer localization). *Cre*-specific primers used in this study were described previously.[8] (B) CRE-mediated excision of the common *Gata4*^{flox} allele is intestine-specific as shown by the absence of a *Gata4*^{flox} exon 2 band in jejunum, but not in heart, of both knock-in mice and het-controls using the F1/F2 primers in semi-quantitative RT-PCR. Amplification of DNA obtained from tail biopsies is shown as a positive control. (C) The region of the *Gata4* cDNAs encompassing the V217G knock-in mutation was amplified by RT-PCR using the Ki3 and Ki4 primers, and sequenced using the nested Ki5 primer. Sequencing of amplified cDNA derived from jejunal RNA verified the presence of the knock-in mutation in knock-in mice, and the wild-type sequence in the het-controls.

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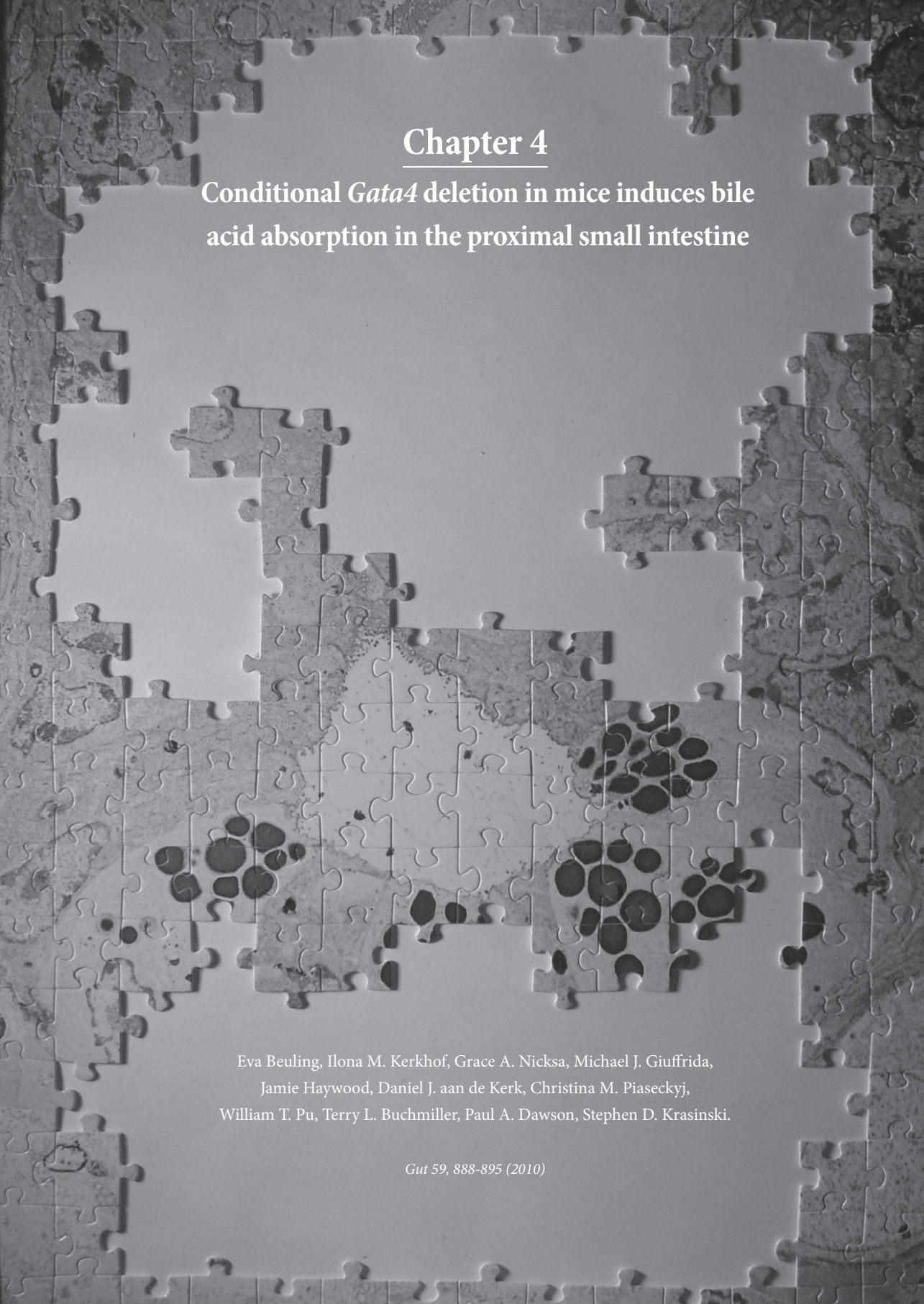

Chapter 4

Conditional *Gata4* deletion in mice induces bile acid absorption in the proximal small intestine

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ABSTRACT

Background & aims: The transcription factor GATA4 is expressed throughout most of the small intestine except distal ileum, and restricts expression of the apical sodium-dependent bile acid transporter (ASBT), the rate-limiting intestinal bile acid transporter, to distal ileum. We tested the hypothesis that reduction of GATA4 activity in mouse small intestine results in an induction of bile acid transport in proximal small intestine sufficient to restore bile acid absorption and homeostasis after ileocecal resection (ICR). **Methods:** Bile acid homeostasis was characterized in nonsurgical, sham, or ICR mice using two recombinant *Gata4* models in which *Asbt* expression is induced to different levels. **Results:** Reduction of intestinal GATA4 activity resulted in an induction of ASBT expression, bile acid absorption, and expression of bile acid-responsive genes in proximal small intestine, and a reduction of luminal bile acids in distal small intestine. While fecal bile acid excretion and bile acid pool size remained unchanged, the bile acid pool became more hydrophilic due to a relative increase in tauro-β-muricholate absorption. Furthermore, proximal induction of *Asbt* in both *Gata4* mutant models corrected ICR-associated bile acid malabsorption, reversing the decrease in bile acid pool size and increase in fecal bile acid excretion and hepatic cholesterol 7α-hydroxylase expression. **Conclusions:** Reduction of intestinal GATA4 activity induces bile acid absorption in proximal small intestine without inducing major changes in bile acid homeostasis. This induction is sufficient to correct bile acid malabsorption caused by ICR in mice.

INTRODUCTION

Ileal diseases and resections result in bile acid malabsorption due to loss of intestinal bile acid transport capacity.[1-3] Bile acid malabsorption is associated with diarrhea, steatorrhea, fat soluble vitamin deficiencies,[2] hyperoxaluria and kidney stones,[4] and gallstone complications,[5] and may be a risk factor for developing colorectal cancer[6-8] and osteoporosis.[9] Current therapies generally focus on sequestering luminal bile acids to relieve bile acid-induced diarrhea[10] or bile acid replacement therapy to reverse the steatorrhea and improve nutrient absorption.[11-13] However, these treatments do not correct bile acid malabsorption, and have only met with limited success.

Recently, we[14] and others[15] demonstrated that GATA4, a member of the GATA family of transcription factors that is expressed throughout most of the small intestine except distal ileum, plays an important role in maintaining jejunal-ileal differences in absorptive enterocyte gene expression. Conditional, inducible deletion of the activation domains encoded by *Gata4* (i.e. synthesis of GATA4Δex2),[14] or conditional deletion of *Gata4*,[15] transforms the expression of specific absorptive enterocyte genes from a jejunal to ileal pattern. One specific change is the induction in the jejunum of the expression of the apical sodium-dependent bile acid transporter (ASBT), the ileal-specific, rate-limiting transporter for bile acid absorption. Based on this finding, we hypothesized that intestinal GATA4 is necessary for limiting *Asbt* gene expression and bile acid absorption to distal ileum, and that an induction of *Asbt* gene expression and bile acid absorption in proximal small intestine by conditionally mutating or deleting *Gata4* is sufficient to correct bile acid malabsorption resulting from ileocecal resection (ICR).

MATERIALS AND METHODS

Mice

Previously, we established a conditional, inducible *Gata4* mutation model[14] in which GATA4 activity in the small intestine is reduced but not eliminated (Model 1, *G4Δex2*, Supplementary Figure 1). To inducibly delete *Gata4* in the mature small intestine, we established a new model (Model 2, *G4ap*, Supplementary Figure 1) using a previously published modification of the *Gata4* allele.[16] In both models, conditional recombination was targeted to the small intestinal epithelium by the Villin*Cre*ER^{T2} transgene.[14, 17, 18] To induce recombination, and also to control for potential tamoxifen effects, all animals were treated with tamoxifen (Sigma-Aldrich, St. Louis, MO) as described.[14, 17] Tissue was collected 14-17 days after the last tamoxifen injection. All mice were previously backcrossed into the C57BL/6 genetic background and only adult (6-12 wk of age) males were used for study. Mice were fed ad libitum standard rodent chow containing approximately 5% w/w fiber and 60% carbohydrate, 28% protein, and 12% fat (% of calories), unless indicated otherwise. Genotypes were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) using previously validated primers (Supplementary Figure 2).[14, 16, 18-20] The genotypes for all

control and test mice are indicated in Supplementary Table 1. Approval was obtained from the Institutional Animal Care and Use Committee.

RNA isolation and reverse transcriptase polymerase chain reaction

Mice were dissected and RNA was isolated from liver and small intestine as described previously.[14] For RNA isolation from small intestine, the location of the tissue samples (approximately 1.0 cm of small intestine) are depicted as gray boxes in Figure 1A and correspond to 5 equidistant regions along the length of the small intestine. The small intestinal tissue samples designated 3 and 5 that were used for RNA isolation are also referred to as jejunum and ileum, respectively. Messenger RNA (mRNA) abundances were determined by semi-quantitative and real-time RT-PCR[14] using validated primer pairs (Supplementary Figure 2 and 3). Glyceraldehyde-3-phosphate dehydrogenase mRNA abundance was measured for each sample and used to normalize the data.

Protein extracts and immunoblotting

After intestinal samples were obtained for RNA isolation, brush border membrane vesicles (BBMV) were prepared as described by Kessler et al[21] using tissue samples I to IV, which correspond to the four quartiles of small intestine (Figure 1A). Western analysis was conducted[22] using 30 µg of BBMV. Primary antibodies included rabbit anti-ASBT[23] (1:3750) and mouse anti-β-actin (1:4000, Sigma).

Bile acid measurements

Mucosal-to-serosal transfer of radiolabelled taurocholate was measured in quartiles I - IV of small intestine (depicted as white boxes in Figure 1A) using everted gut sacs as previously described.[24] Bile acid concentrations in stools from 72 h collections, and also in segmental luminal contents were determined as previously described.[24-26] Bile acid pool size and composition was determined from liver, gall bladder and small intestine as previously described.[24] In selected experiments, bile acid composition was also determined in luminal contents. The bile acid hydrophobicity index was calculated according to Heuman.[27]

Surgery

Ten to twelve week old male mice were transferred to a liquid rodent diet (Bio-serve Inc, Frenchtown, NJ) two days prior to surgery. Mice were weighed and sham or ICR surgery was performed as previously described[28] (Supplementary Figure 4). In mice that underwent ICR, ~10 cm of ileum proximal to the ileocecal junction and cecum were removed, and the colon was anastomosed to the remaining small intestine. *Asbt* mRNA abundance in the most proximal one-cm sample of the resected small intestine was low or undetectable, indicating that all of the native *Asbt*-expressing tissue was removed. In mice that underwent sham operations, transection and anastomosis occurred ~10 cm proximal to the ileocecal junction. Mice were given 2 ml intraperitoneal zosyn (100 mg/kg, Wyeth Pharmaceuticals, Philadelphia, PA) in phosphate-buffered saline and the abdomen

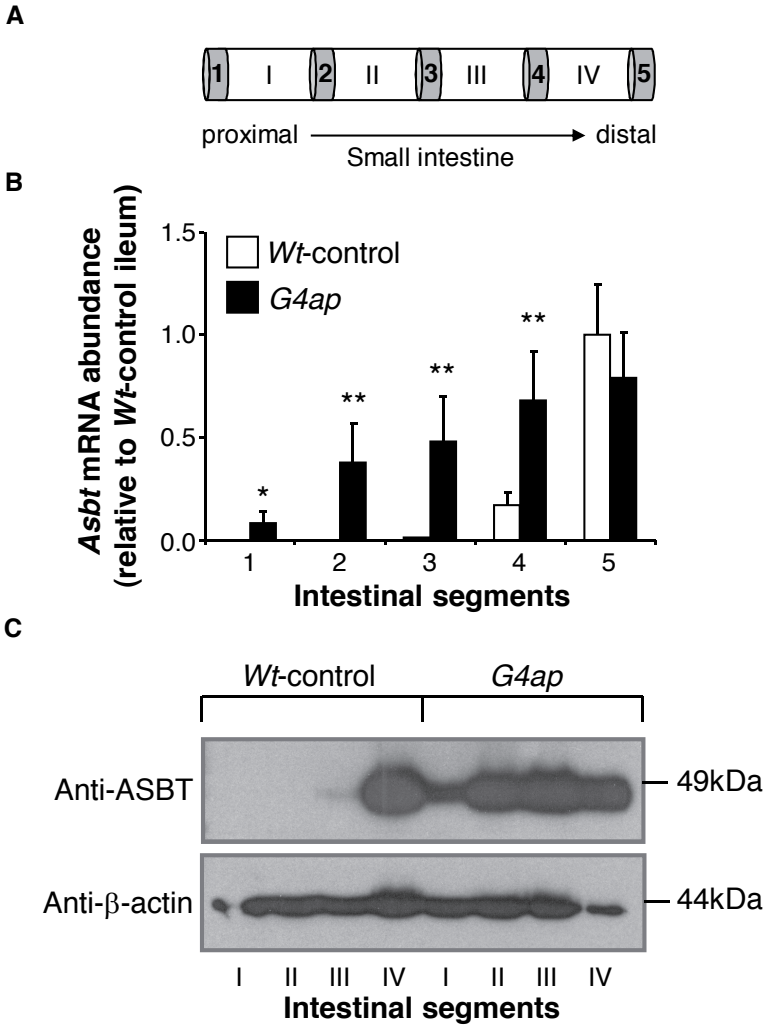

Figure 1. Intestinal *Gata4* deletion results in an induction of ASBT expression in proximal small intestine. (A) Schematic representation of intestinal sampling. The gray bars numbered 1 to 5 indicate the ~1.0 cm segments used for RNA isolation. The white bars with Roman numerals I to IV indicate the ~7 cm segments used for the isolation of brush border membrane vesicles. (B) Real-time RT-PCR reveals a proximal induction of apical sodium-dependent bile acid transporter (*Asbt*) mRNA in *G4ap* mice. *P<0.05, **P<0.01, as compared to *Wt*-controls, n=4 in each group. Values are presented relative to the mean value of the *Wt*-control segment 5 samples. (C) Western analysis shows a proximal induction of ASBT protein in *G4ap* mice. β-actin was used as a loading control.

was closed. The mice were incubated at 27°C for 2-4 h after surgery, and then transferred to their normal housing, with access to liquid diet. After a week of recovery, the mice were transitioned to solid food. Three weeks after surgery, mice were treated with tamoxifen and tissue was collected

as described above. All mice treated with tamoxifen were fully recovered from surgery as indicated by normal activity, weight gain to pre-surgical body weights, and formed stools. The overall mortality rate was 59%; virtually all mice that did not recover from surgery died of obstruction.

Statistical analyses

Data are expressed as mean ± SD. Statistically significant differences were determined by the two-tailed Student's *t* test or analysis of variance followed by the Tukey-Kramer multiple comparison test. Differences were considered statistically significant at $P<0.05$.

Expanded methods

More detailed description of all methodology is provided in a supplementary "Expanded Methods Section".

RESULTS

Intestinal *Gata4* deletion results in a proximal induction of bile acid absorption

Conditional deletion of *Gata4* in our new *G4ap* model (Model 2, Supplementary Figure 1) was virtually complete, as indicated by the elimination of *Gata4* mRNA and GATA4 protein (Supplementary Figure 5). Body weights (BW), plasma triglycerides and plasma cholesterol were not different between *Wt*-control and *G4ap* mice (Supplementary Table 2). *Asbt* mRNA (Figure 1B) and protein (Figure 1C), normally restricted to the distal ileum, were significantly induced throughout the small intestine of *G4ap* mice suggesting that the proximal small intestine acquires the underlying capacity to absorb bile acids.

Taurocholate (TC) transport to the serosal fluid (Figure 2A) and tissue-associated TC (Figure 2B) in everted gut sacs were both significantly increased in proximal segments after intestinal *Gata4* deletion, demonstrating directly that this region acquires the capacity to take up and transport bile acids. Bile acid excretion from a 3-day stool collection was not significantly different between *Wt*-control and *G4ap* mice (9.6 ± 3.6 versus 12.3 ± 1.1 $\mu moles \cdot day^{-1} \cdot 100g^{-1}$ BW, n=5 in each group), indicating that intestinal *Gata4* deletion does not induce major changes in overall bile acid absorption in vivo. Bile acid concentrations in luminal contents (Figure 2C) and tissue (Figure 2D) in *Wt*-control mice were similar among all four segments of small intestine, consistent with minimal bile acid absorption throughout most of the small intestine. In contrast, luminal and tissue-associated bile acids in *G4ap* mice were high proximally and decreased distally. The increased levels of bile acids in the luminal contents of proximal intestine after *Gata4* deletion may be due to decreased intestinal motility. Inactivation of *Gata4* results in increased proximal expression of peptide YY,[14, 15] the ileum-derived peptide hormone that is partially responsible for mediating the "ileal brake", the inhibitory feedback mechanism that slows transit of a meal

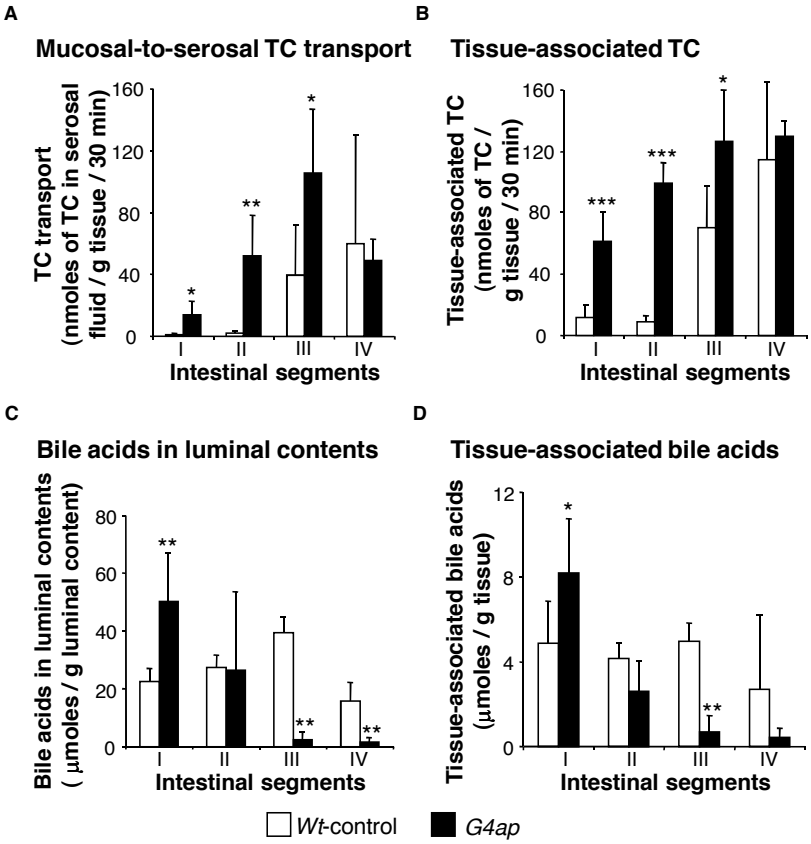

Figure 2. Intestinal *Gata4* deletion results in an induction of taurocholate uptake in proximal small intestine, and a depletion of luminal bile acids in distal small intestine. Ex vivo measurements of taurocholate (TC) transport in everted gut sacs shows (A) a significant increase in mucosal-to-serosal transport of radioactively labelled TC in proximal intestine of *G4ap* mice as compared to *Wt*-controls, and (B) a corresponding increase in tissue-associated TC. *P<0.05, **P<0.01, ***P<0.001, as compared to *Wt*-controls, n=5 in each group. In vivo segmental analysis reveals that the amount of bile acid in luminal contents (C) and tissue (D) is reduced in distal small intestine of *G4ap* mice as compared to *Wt*-controls. *P<0.05, **P<0.01, as compared to *Wt*-controls, n=6 for *G4ap* mice, n=5 for *Wt*-control mice.

through the gastrointestinal tract.[29] The nearly complete depletion of bile acids in distal small intestine is consistent with an induction of bile acid absorption in the proximal small intestine.

Intestinal *Gata4* deletion results in altered patterns of expression of intestinal bile acid-responsive genes

We next examined the expression of known bile acid-responsive genes, including the cytosolic bile acid binding protein (ileal lipid binding protein; *Ilbp*), subunits of the basolateral membrane bile acid transporter (organic solute transporter alpha-beta; *Ost*α-*Ost*β), and fibroblast growth

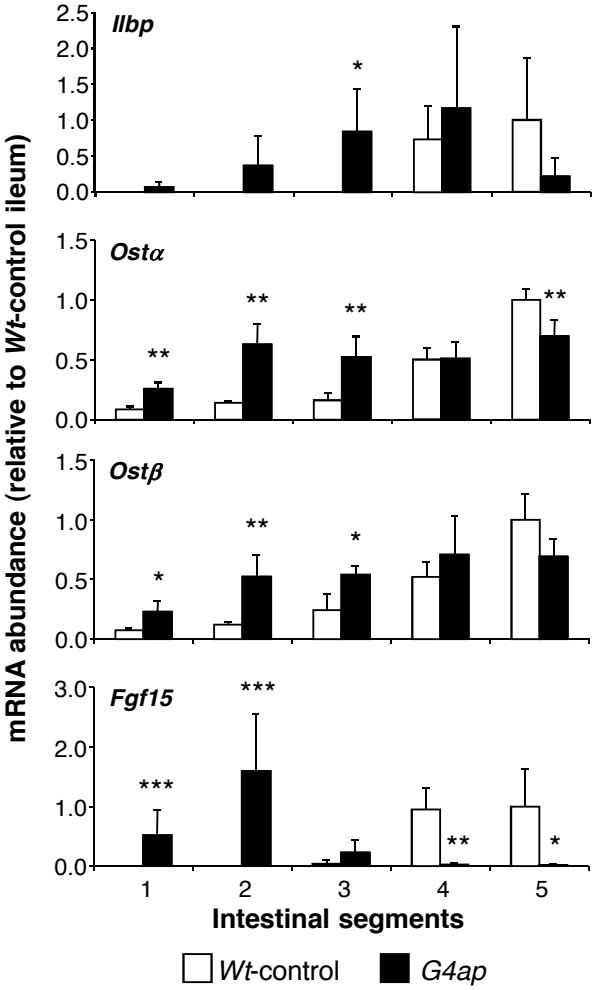

Figure 3. Intestinal *Gata4* deletion results in an increase in proximal and a decrease in distal expression of intestinal genes regulated by bile acids. Real-time RT-PCR analyses of RNA from intestinal segments along the length of the small intestine show a general increase in ileal lipid binding protein (*Ilbp*), organic solute transporter (*Ost*)*α* and *β*, and fibroblast growth factor 15 (*Fgf15)* mRNA abundances in proximal segments, and decrease in distal segments of *G4ap* mice as compared to *Wt*-controls. *P<0.05, **P<0.01, ***P<0.001, as compared to *Wt*-controls, n=4 in each group. Values are presented relative to the mean value of the *Wt*-control segment 5 samples.

factor-15 (*Fgf15*), an intestine-derived regulator of hepatic bile acid synthesis. As shown in Figure 3, the expression of *Ilbp*, *Ost*α, *Ost*β and *Fgf15* is normally most abundant in distal small intestine, but was induced (in most cases significantly) in proximal segments of small intestine after *Gata4* deletion. The expression of these genes tended to be lower in distal small intestine of *G4ap* mice as compared to *Wt*-control mice, with *Ost*α and *Fgf15* attaining statistical significance. These

patterns are consistent with an increase in bile acid flux in absorptive enterocytes in proximal segments and a decrease in bile acid flux in distal segments. *Fgf15* mRNA abundance was induced proximally and reduced to undetectable levels in distal segments demonstrating a high sensitivity to bile acid flux across the absorptive enterocyte.

Intestinal *Gata4* recombination results in tauro-β-muricholate enrichment of the bile acid pool

As shown in Figure 4A, deletion of the activation domains of *Gata4* in our previously characterized model (*G4Δex2*)[14] resulted in an induction of *Asbt* mRNA in jejunum that is 22% of *Wt*-control ileum levels (low *Asbt* induction), whereas deletion of *Gata4* in our new model (*G4ap*) resulted in an induction that is 69% of *Wt*-control ileum levels (high *Asbt* induction). The graded responses of these two mouse lines was also confirmed for lactase phlorizin hydrolase (*Lph*) (Supplementary Figure 6), an activation target of GATA4.[14] Characterization of both models, therefore, allows us to define the effects of low versus high proximal induction of *Asbt*.

As shown in Figure 4B, the bile acid pool size did not differ among *Wt*-control, *G4Δex2* and *G4ap* mice, indicating that induced bile acid absorption in the proximal small intestine does not alter the size of the bile acid pool. Surprisingly, however, the bile acid pool composition became progressively enriched in tauro-β-muricholate (TBMC) in *G4Δex2* and *G4ap* mice (Figure 4B), resulting in a significant increase in the TBMC/TC ratio (Figure 4C). Because TBMC is more hydrophilic than TC,[27] the hydrophobicity index of the bile acid pool was significantly decreased in *G4Δex2* and *G4ap* mice (Figure 4D).

Tauro-β-muricholate enrichment of the bile acid pool after intestinal *Gata4* deletion is due to a relative increase in tauro-β-muricholate uptake by the small intestine

To understand the mechanism underlying the change in pool composition, we characterized potential alterations is bile acid biosynthesis in *Wt*-control and *G4ap* mice. Surprisingly, none of the mRNAs for key enzymes in the hepatic bile acid biosynthetic pathways (cholesterol 7α-hydroxylase (CYP7a1),[30,31] sterol 27-hydroxylase (CYP27),[32] sterol 12α-hydroxylase (CYP8b1),[33] and oxysterol 7α-hydroxylase (CYP7b1)[34]) were significantly different in the *G4ap* mice as compared to *Wt*-controls (Figure 5A), suggesting that the shift in bile acid composition is not due to alterations in bile acid synthesis.

Since studies of the bile acid substrate specificity of ASBT revealed a marked preference for TC as compared to the taurine-conjugated forms of α, β, or ω-muricholic acid,[35] we hypothesized that TC but not TBMC absorption is almost complete from terminal ileum in the wild type situation, and that the shift in the bile acid pool composition is determined by enhanced absorption of TBMC as a result of increased total intestinal ASBT expression. To test this hypothesis, we assessed the TBMC/TC ratio in luminal contents along the length of the small intestine of *Wt*-control and *G4ap* mice. The TBMC/TC ratio in segment I was 3.4-fold higher in *G4ap* mice (2.68 ± 1.07) as compared to *Wt*-controls (0.84 ± 0.39) ($P<0.05$), demonstrating that the composition

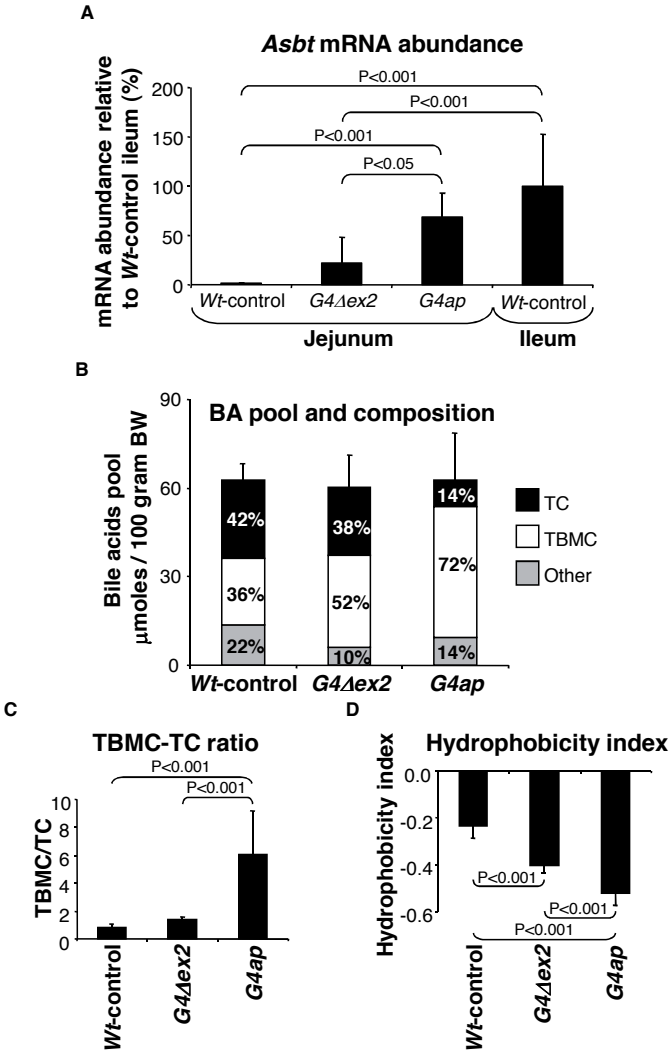

Figure 4. Intestinal *Gata4* recombination results in an enrichment of tauro-β-muricholate and reduction of taurocholate in the bile acid pool. (A) Real-time RT-PCR of apical sodium-dependent bile acid transporter (*Asbt*) mRNA in *Wt*-control jejunum (segment 3) and ileum (segment 5), *G4Δex2* jejunum, and *G4ap* jejunum, shows a 22% and 69% transformation to wild-type ileal levels in jejunum of *G4Δex2* and *G4ap* mice, respectively. n=6-10 in each group. (B) Bile acid pool size, as determined by the total bile acid content in liver, gall bladder and small intestine, is similar among Wt-control, *G4ex2* and *G4ap* mice, but demonstrates an increase in tauro-β-muricholate (TBMC), and decrease in taurocholate (TC) and other bile acids in *G4ex2* and *G4ap* mice as compared to *Wt*-controls. (C) The TBMC/TC ratio of the bile acid pool reveals a 2.2-fold increase in *G4ex2* mice and a 7.2-fold (P<0.001) increase in *G4ap* mice as compared to *Wt*-controls. (D) The hydrophobicity index of the bile acid pool reveals a 1.7-fold (P<0.001) decrease in *G4ex2* mice as compared to *Wt*-controls. (n=4-7 in each group).

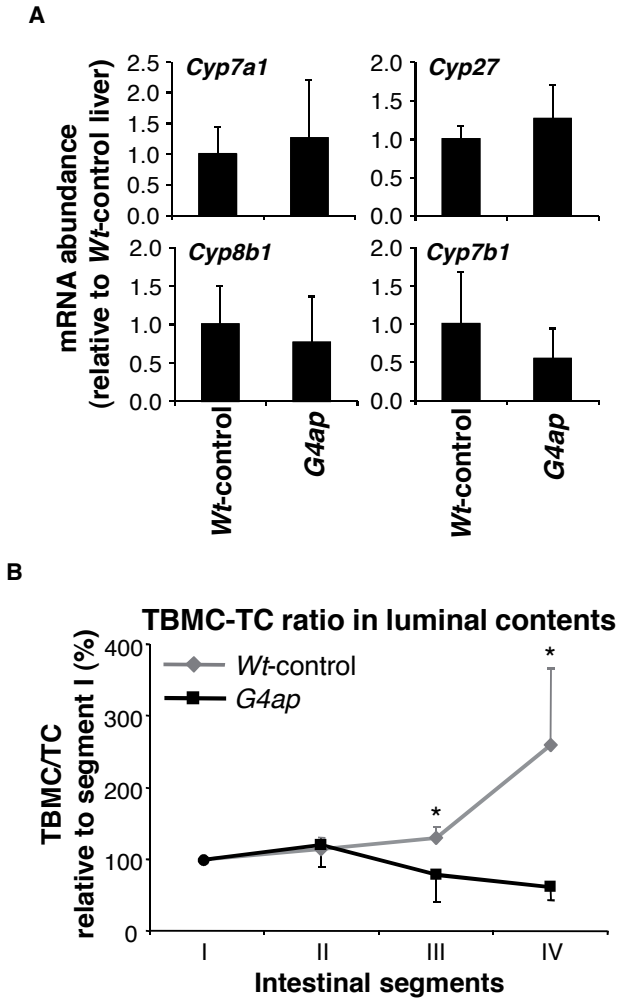

Figure 5. Tauro-β-muricholate-enrichment of the bile acid pool after intestinal *Gata4* deletion is due to an increase in tauro-β-muricholate uptake. (A) Real-time RT-PCR shows that the mRNAs for hepatic bile acid biosynthetic enzymes, *Cyp7a1*, *Cyp8b1*, *Cyp27* and *Cyp7b1,* are similar between *Wt*-control and *G4ap* mice (n=5 in each group). Values are presented relative to the mean value of *Wt*-control liver samples. (B) Segmental analysis of the bile acid composition of luminal contents reveals that the tauro-β-muricholate/taurocholate (TBMC/TC) ratio as a percentage of segment I increases distally in *Wt*-controls, but decreases distally in the *G4ap* mice. *P<0.05 as compared to *Wt*-controls, n=3-6 in each group.

of the bile acids secreted into the duodenum by the gall bladder is already TBMC-enriched. As shown in Figure 5B, the TBMC/TC ratio in the luminal contents of *Wt*-control mice progressively increased distally approaching a 3-fold rise from segment I, II, or III to segment IV, reflecting a more efficient absorption of TC than TBMC in this limited region of distal small intestine where ASBT is expressed. In contrast, the TBMC/TC ratio in the luminal contents of *G4ap* mice de-

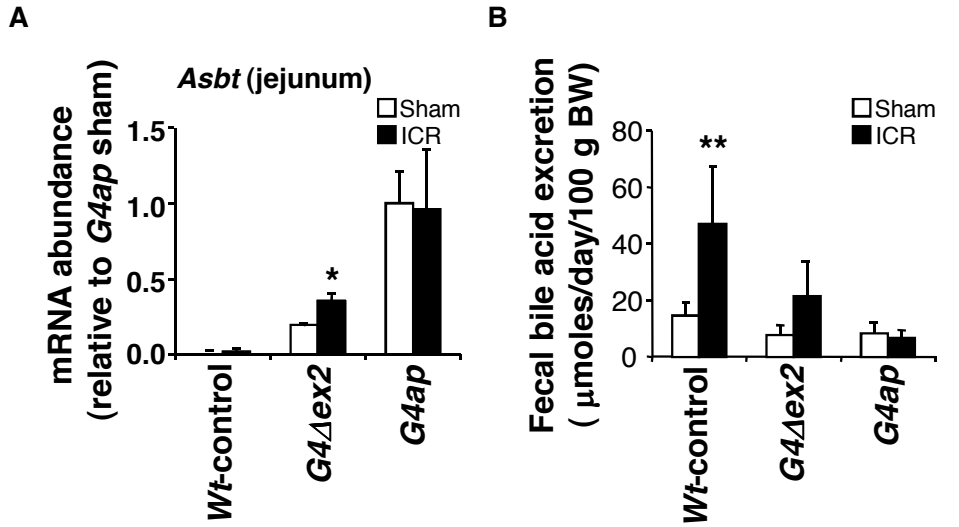

Figure 6. Intestinal *Gata4* recombination restores bile acid absorption after ICR. (A) Real-time RT-PCR on RNA obtained from jejunum shows an induction of the apical sodium-dependent bile acid transporter (*Asbt*) mRNA in sham-operated and ICR *G4Δex2* and *G4ap* mice. Values are presented relative to the mean value of the sham-operated *G4ap* samples. *P<0.05 as compared to sham-operated mice, n=3-7 in each group. (B) Fecal bile acid content is increased in *Wt*-control ICR mice as compared to their sham-operated counterparts, but approached or returned to normal levels in *G4Δex2* and *G4ap* mice that underwent ICR. **P<0.01 as compared to sham-operated mice, n=3-7 in each group.

creased distally, and was significantly lower than *Wt*-controls in the distal half (segments III & IV, Figure 5B), revealing that when ASBT is expressed over the total length of the small intestine, a greater proportion of TBMC is absorbed in the *G4ap* mice as compared to *Wt*-controls.

Intestinal *Gata4* recombination corrects bile acid malabsorption associated with ileocecal resection

To determine if proximal induction of bile acid absorption compensates for bile acid malabsorption due to a loss of ileal function, sham or ICR surgery was performed. As shown in Figure 6A, *Asbt* mRNA was incrementally induced in *G4Δex2* and *G4ap* jejunum (See Supplementary Figure 4 for sampling location) of both sham and ICR mice, with an increase similar to that found in non-operated animals of the respective genotypes (see Figure 4A), demonstrating that sham or ICR surgery does not affect the proximal induction of *Asbt*. Fecal bile acid excretion in *Wt*-control ICR mice was significantly higher than in sham-operated mice (3.2-fold, P<0.01) (Figure 6B), with 6 of 7 excreting more than 25 µmoles·day^{-1}·100g^{-1} BW, confirming malabsorption of bile acids.[1, 36, 37] In *G4Δex2* ICR mice, bile acid excretion was higher than in sham-operated mice (2.8-fold, P=0.11), but only 1 of 4 excreted more than 25 µmoles·day^{-1}·100g^{-1} BW. Fecal bile acid excretion in *G4ap* ICR mice was similar to sham-operated mice, and no excretion value (n=6) was

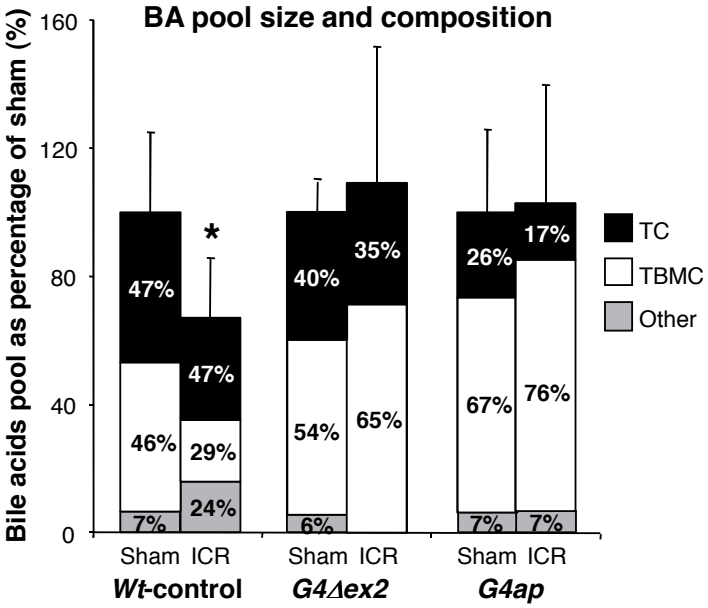

Figure 7. Intestinal *Gata4* recombination prevents a loss of bile acids in the body pool after ICR. Bile acid pool size, as determined by the total bile acid content in liver, gall bladder and small intestine, is lower in *Wt*-control mice that underwent ICR as compared to sham-operated mice. Bile acid pool size in *G4Δex2* and *G4ap* mice that underwent ICR remains similar to their sham-operated counterparts. *P<0.05, as compared to sham-operated mice, n=3-7 in each group.

above 25 μmoles·day^{-1}·100g^{-1} BW. These data demonstrate that low (*G4Δex2*) or high (*G4ap*) *Asbt* induction results in a partial or complete rescue of bile acid absorption, respectively.

As shown in Figure 7, the whole body bile acid pool size was 33% lower in *Wt*-control ICR mice as compared to sham-operated mice, reflecting bile acid malabsorption and an inability of de novo hepatic synthesis to compensate for bile acid loss. In contrast, the bile acid body pool was not decreased by ICR in *G4Δex2* and *G4ap* mice as compared to their sham counterparts, suggesting that the proximal induction of bile acid absorption in both *Gata4* mutant models is sufficient to maintain an efficient enterohepatic circulation of bile acids. Noteworthy, in *Wt*-control mice, ICR resulted in an increase in the proportion of TC plus TC metabolite (taurodeoxycholate) and a relative decrease in TBMC in the bile acid pool. This alteration in composition is consistent with an ICR-induced increase in hepatic synthesis of TC via the CYP7A1/CYP8B1 pathway and increased TC spillage into the colon, where it is converted to deoxycholate (DC) by the bacterial flora and partially reabsorbed.[38, 39] No increase of DC was found in *G4Δex2* and *G4ap* mice after ICR, suggesting that TC is being absorbed in the proximal small intestine, before encountering the bacterial flora in the distal small intestine and colon.

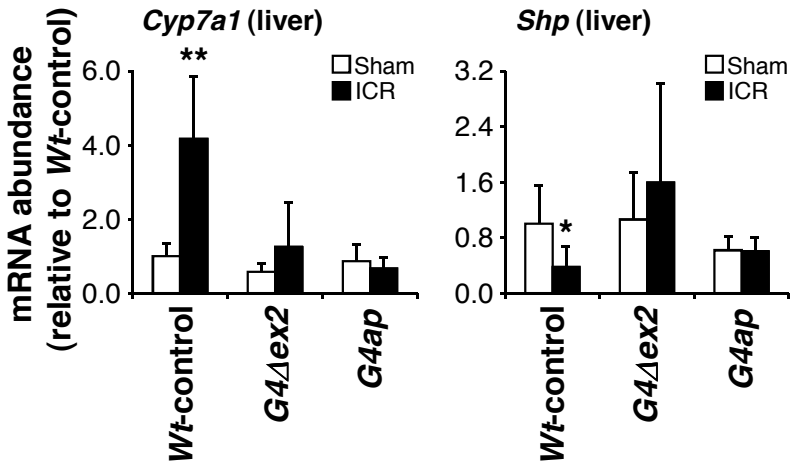

Figure 8. Intestinal *Gata4* recombination eliminates the need for compensatory up-regulation in bile acid synthesis after ICR. Real-time RT-PCR shows an increase in *Cyp7a1* and a decrease in *Shp* mRNA abundance in liver of *Wt*-control mice that underwent ICR as compared to sham-operated mice, whereas no major differences were found between sham-operated and ICR *G4Δex2* and *G4ap* mice. *P<0.05, **P<0.01, as compared to sham-operated mice, n=3-7 in each group. Values are presented relative to the mean value of the sham-operated *Wt*-control liver samples.

In *Wt*-controls, ICR resulted in a 4.2-fold increase in *Cyp7a1* mRNA (P<0.01) and a 2.7-fold decrease in small heterodimer partner (*Shp*) mRNA (P<0.05) in liver as compared to sham-operated mice (Figure 8), consistent with a compensatory up-regulation of bile acid synthesis in response to bile acid malabsorption.[40] Neither an increase in *Cyp7a1* mRNA nor a decrease in *Shp* mRNA was found in *G4ap* mice after ICR, suggesting that proximal bile acid uptake in *Gata4* mutant mice is sufficient to maintain bile acid homeostasis.

DISCUSSION

GATA4, a member of the evolutionarily conserved GATA transcription factor family, exhibits critical and diverse functions in cellular proliferation, differentiation and gene regulation in multiple organs.[41] Using an in vivo model in which the activation domains of *Gata4* are deleted, we were the first to show that GATA4 determines key differences in absorptive enterocyte gene expression between jejunum and ileum.[14] Here, we show that intestinal *Gata4* deletion results in an induction of *Asbt* gene expression and bile acid absorption in the proximal small intestine that is sufficient to restore bile acid absorption, bile acid pool size, and hepatic *Cyp7a1* mRNA abundance to physiological levels after ICR, without major defects in bile acid homeostasis.

Induction of intestinal *Asbt* expression and bile acid absorption has been previously reported. Treatment with glucocorticoid hormones precociously induces ASBT and ILBP expression in the

distal small intestine during development,[42-44] but the effect on proximal small intestine was not reported. Compensatory changes in ASBT expression have been shown in adult rats after ileal resection or transposition,[40, 45, 46] but these changes occur only in regions of the small intestine that natively express ASBT, and are not sufficient to correct bile acid malabsorption after ileoectomy.[40] Transplantation of ileal neonatal rat stem cells into adult rat jejunum leads to expression of ASBT and bile acid absorption in this segment, and reduces bile acid excretion to physiological levels after ileoectomy,[47] demonstrating that ileal stem cells maintain their ileal character, and thus likely give rise to enterocytes that do not express GATA4. Our study is the first to demonstrate an induction of ASBT expression and bile acid absorption in enterocytes that normally do not exhibit these characteristics.

While absorption of bile acids in proximal small intestine does not result in alterations in fecal bile acid excretion, overall pool size (Figure 4A), BW, and plasma triglyceride or cholesterol levels (Supplementary Table 2), our data show that proximal absorption of bile acids results in TBMC-enrichment of the bile acid pool (Figure 4B). An increase in the TBMC fraction of the bile acid pool was also found in *Cyp8b1*[33] and liver receptor homolog-1[48, 49] knockout mice. In both models, CYP8b1 is eliminated or dramatically reduced, leading to an increase in muricholic acid synthesis, and accumulation of TBMC in the bile acid pool. Surprisingly, in our model, the TBMC-enrichment of the pool was not due to alterations in the expression of hepatic bile acid biosynthetic enzymes (Figure 5A), but resulted from the cumulative increase in TBMC uptake by the small intestine relative to TC (Figure 5B). ASBT has a higher affinity for TC than TBMC,[35] and as such the absorption of TC but not TBMC is almost complete from terminal ileum in the wild type situation (Figure 5B). By inducing ASBT expression in the proximal small intestine, the opportunity for TBMC uptake is increased leading to a cumulative increase in the TBMC fraction in the pool over many enterohepatic cycles. Accordingly, our data demonstrate that the restriction of ASBT and active bile acid absorption to the distal small intestine by GATA4 plays a key role in determining the composition of the bile acid pool.

Using ICR in mice[28] to induce bile acid malabsorption, we show that resection of terminal ileum and cecum leads to a marked increase of fecal bile acid excretion (Figure 6B), a reduction of the bile acid pool (Figure 7) and a compensatory up-regulation of the expression of hepatic *Cyp7a1* mRNA (Figure 8), hallmarks of bile acid malabsorption reported in humans[1] and rats[40] after ileoectomy. We show here that low or high induction of *Asbt* expression by modulating intestinal GATA4 activity induces bile acid absorption in proximal small intestine that is sufficient to reduce bile acid excretion (Figure 6B), maintain the bile acid pool size (Figure 7), and reduce the compensatory up-regulation of bile acid synthesis (Figure 8) after ICR. This is the first example of a non-transplant intervention able to restore intestinal bile acid absorptive function following ileoectomy.

Battle et al[15] showed that conditional *Gata4* deletion in the mouse small intestine results in cholesterol and fat malabsorption, and attributed this to a decrease in the expression of specific jejunal genes that encode proteins involved in these processes. It is also possible that depletion of

luminal bile acids by the proximal induction of *Asbt* (Figure 2C) results in inefficient solublization and absorption of lipids and cholesterol. Further, since bile acid feeding experiments have shown that TBMC causes a decrease in cholesterol absorption in the small intestine of mice,[50] TBMC-enrichment of the bile acid pool could contribute to reduced cholesterol absorption. Thus, the underlying cause of reduced fat and cholesterol absorption in these models remains to be determined.

In patients with mild-to-moderate ileal disease or limited ileal resection, bile acid malabsorption leads to diarrhea due to increased concentrations of bile acids in the colon.[51] With extensive ileal disease or resection, maldigestion of fat is caused by a decrease in bile acid secretion into the small intestine due to a reduced bile acid pool.[1] Current therapies focus on the symptoms associated with bile acid malabsorption, but do not correct the bile acid malabsorption itself. Our data show that a reduction of intestinal GATA4 activity induces bile acid absorption in proximal small intestine that is sufficient to restore bile acid absorption and maintain the bile acid pool after ICR in mice. Although the effect of the proximal absorption of bile acids on fat and protein digestion and absorption, the intestinal flora, and gene regulation,[52] as well as the effect of inducing ileal function and reducing jejunal function by decreasing GATA4 activity remain to be determined, inducing ASBT expression in the proximal small intestine by reducing GATA4 activity may be useful in the development of therapeutic interventions for patients with bile acid malabsorption due to ileal disease or resection. An approach in which *Gata4* is only partially inactivated, as in our *G4Δex2* model, or by disrupting interactions between GATA4 and friend of GATA (FOG), which results in a modest jejunal induction of *Asbt* expression,[17] might be desirable. In these situations, ASBT may be induced in the proximal small intestine to levels that restore bile acid absorption and pool size to homeostasis after ICR, but minimize potential negative side effects that may arise from the complete inactivation of *Gata4*.

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GRANTS

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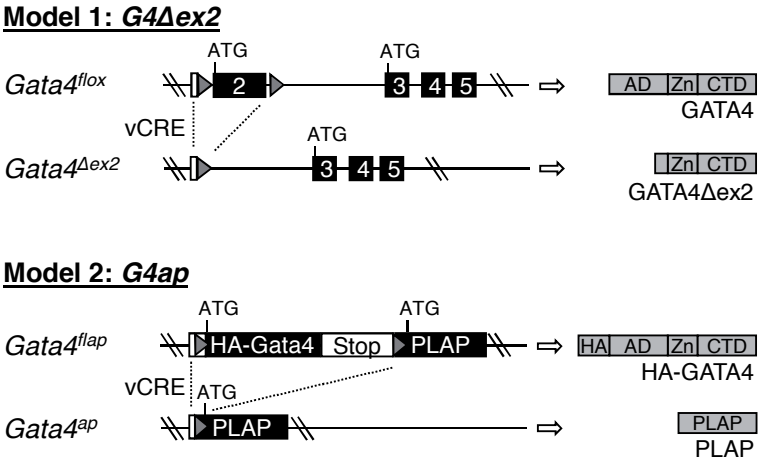
SUPPLEMENTAL FILES



Supplementary Figure 1. Schematic representation of conditional Gata4 recombination models. In Model 1, the *Gata4*^{flox} allele, in which exon 2 is flanked by loxP sites (gray arrowheads), expresses WT GATA4 under control of the endogenous *Gata4* promoter. After intestine-specific CRE-excision, mediated by the Villin*Cre*ER^{T2} transgene (vCRE), the *Gata4*^{flox} allele is recombined (Gata4Δex2), resulting in deletion of the GATA4 activation domains and the expression of a truncated form of Gata4 (GATA4ex2) in which an in-frame ATG in exon 3 is utilized. In Model 2, the *Gata4*^{flap} allele contains a cDNA that encodes GATA4 tagged with influenza hemaglutinin (HA). After intestine-specific CRE excision, the *Gata4*^{flap} allele is recombined (*Gata4*^{ap}), resulting in excision of *HA-Gata4* and the expression of placental alkaline phosphatase (PLAP) under the control of the endogenous *Gata4* promoter. Filled boxes with numbers indicate exons. Grey boxes indicate peptides. AD, activation domains; Zn, zinc fingers; CTD, C-terminal domain.

Genotyping primers:

Cre:	F: 5'-CGTATAGCCGAAATTGCCAG-3'
	R: 5'-CAAAACAGGTAGTTATTCGG-3'
Gata4:	
Flox1:	F: 5'-GGTGGTTTCATTTGCTGTGTGGAAG-3'
Flox2:	R: 5'-AATCGTGCGGGAGGGCGGACTCTATTC-3'
Flap1:	F: 5'-CTTCGACAGCCCAGTCCTGCAC-3'
Flap2:	R: 5'-GCACAGGTAGTGTCCCGTCCCATC-3'
Flap3:	R: 5'-ACCAGTAGGCTACCCAGACATTGCTGG-3'

Semi-quantitative RT-PCR primers:

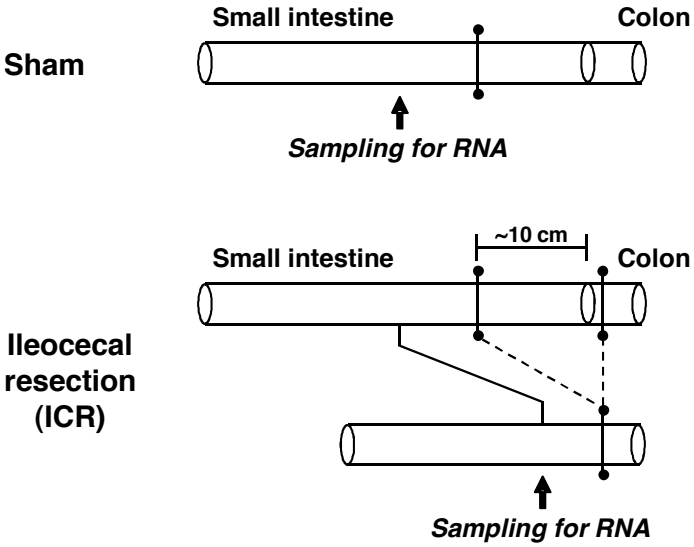
Gata4:	F: 5'-AACCCTGGAAGACACC-3'
	R: 5'-CATTGCTGGAGTTACCG-3'
Gapdh:	F: 5'-GCCTTCCGTGTTCCTACCC-3'
	R: 5'-TGCCTGCTTCACCACCTTC-3'

Supplementary Figure 2. Genotyping and semi-quantitative RT-PCR primer sequences.

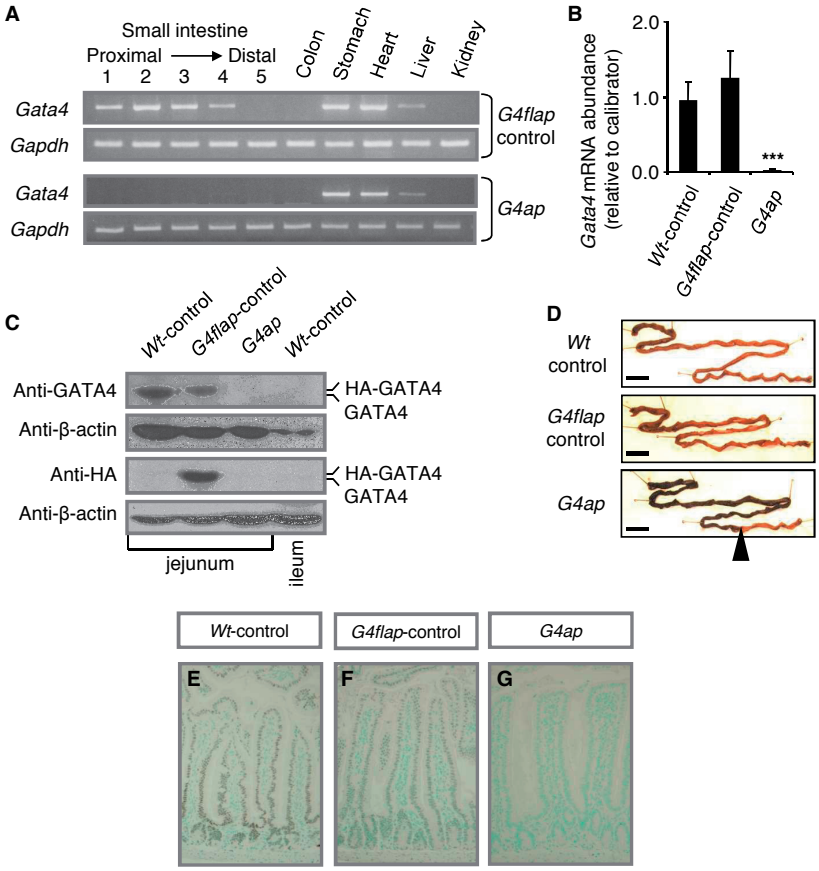
Real time RT-PCR primers:

Asbt:	F: 5'-TTGCCTCTTCGTCTACACC-3'
	R: 5'-CCAAAGGAAACAGGAATAACAAG-3'
Ilbp:	F: 5'-TGGCAAAGAATGTGAAATG-3'
	R: 5'-CTCCGAAGTCTGGTGATAG-3'
Ostα:	F: 5'-TACAAGAACACCCTTTGCCC-3'
	R: 5'-CGAGGAATCCAGAGACCAAA-3'
Ostβ:	F: 5'-GTATTTTCGTGCAGAAGATGCG-3'
	R: 5'-TTTCTGTTTGCCAGGATGCTC-3'
Fgf15:	F: 5'-CCTGTTGTGTTAGTGGCTA-3'
	R: 5'-GAGTAAGTTCCCTATTAGTGG-3'
Cyp7a1:	F: 5'-GCCAGAGTCCAATGCTTAGG-3'
	R: 5'-ATCTCACACCAGGGTAAATGC-3'
Cyp7b1:	F: 5'-TAGCCCTCTTTCCTCCACTCATA-3'
	R: 5'-GAACCGATCGAACCTAAATTCCT-3'
Cyp8b1:	F: 5'-GCCTTCAAGTATGATCGGTTCCT-3'
	R: 5'-GATCTTCTTGCCCGACTTGTAGA-3'
Cyp27a1:	F: 5'-GGAGGGCAAGTACCCAATAAGA-3'
	R: 5'-TGCGATGAAGATCCCATAGGT-3'
Shp:	F: 5'-GCAACAGGAGGCTCACTG-3'
	R: 5'-ATGATAGGGCGGAAGAAGAG-3'
Gata4:	F: 5'-TTTGAGCGAGTTGGG-3'
	R: 5'-GAATGCGGGTGTGC-3'
Lph:	F: 5'-CAGCGATGCCCACAGGAAAG-3'
	R: 5'-ACGGAGCCCTTGACGAGAG-3'
Gapdh:	F: 5'-GCCTTCCGTGTTCCTACCC-3'
	R: 5'-TGCCTGCTTCACCACCTTC-3'

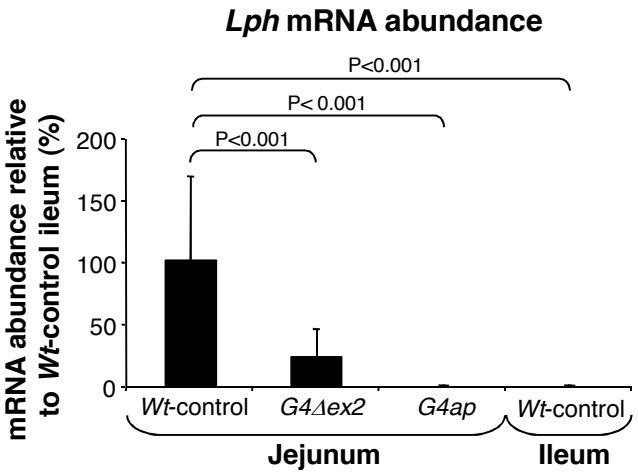
Supplementary Figure 3. Real time RT-PCR primer sequences.



Supplementary Figure 4. Schematic representation of sham and ileocecal resection (ICR) surgery. Arrows depict primary sampling location for RNA analyses.



Supplementary Figure 5. Villin*Cre*ER^{T2}-mediated recombination of the *Gata4^{flap}* allele results in conditional deletion of *Gata4* in the mouse small intestine. (A) Semi-quantitative RT-PCR analysis reveals a normal *Gata4* mRNA expression pattern in a representative *G4flap*-control mouse, and specific deletion of *Gata4* mRNA in the small intestine of a representative *G4ap* mouse. Small intestinal segments 1-5 represent 5 equidistant segments along the length of small intestine, as described in Materials and Methods. (B) Real-time RT-PCR analysis of mouse jejunum (segment 3) shows that *Gata4* mRNA is significantly reduced in *G4ap* mice as compared to *Wt*-control and *G4flap*-control jejunum. ***P<0.001, as compared to all other groups as determined by ANOVA and the Tukey-Kramer multiple comparison test, n=6 in each group. RNA from jejunum of a *Wt*-control mouse was used as the calibrator. (C) Western analysis of nuclear extracts isolated from small intestine demonstrates that GATA4 and HA-GATA4 are expressed in jejunum of *Wt*-control and *G4flap*-control mice, respectively, but neither is expressed in jejunum of *G4ap* mice. *Wt*-control ileum is shown as a negative control. (D) Whole mount staining reveals that placental alkaline phosphatase (PLAP) is expressed in the proximal 85 ± 2% (n=3) of small intestine of *G4ap* mice, but is not expressed in *Wt*-control and *G4flap*-control mice, confirming that the *Gata4^{flap}* alleles are recombined specifically in the small intestine of *G4ap* mice. Arrowhead indicates the demarcation between the PLAP-expressing and PLAP-non-expressing region. (E-G) Immunostaining for GATA4 of *Wt*-control (E), *G4flap*-control (F) and *G4ap* (G) jejunum shows absence of GATA4 in the *G4ap* mice.



Supplementary Figure 6. Real-time RT-PCR of lactase phlorizin hydriolase (*Lph*) mRNA in *Wt*-control jejunum (segment 3) and ileum (segment 5), *G4Δex2* jejunum, and *G4ap* jejunum, shows a partial and more complete transformation to wild-type ileal levels in the jejunum of *G4Δex2* and *G4ap* mice, respectively. P<0.001, as compared to *Wt*-control jejunum, n= 6-10 in each group.

Supplementary Table 1 . Nomenclature for the different mouse lines used in this study.

Nomenclature	*Gata4* alleles	Villin*Cre*ER^{T2}	Tamoxifen
Wt-control	*Gata4*$^{wt/wt}$	positive	treated
G4Δex2	*Gata4*$^{flox/flox}$	positive	treated
G4ap	*Gata4*$^{flap/flap}$	positive	treated

Supplementary Table 2. Body weights, plasma triglycerides and plasma cholesterol in *Wt*-control and *G4ap* mice.

	Wt-control	*G4ap*
Body weights (g)	28.0 ± 1.8	25.7 ± 2.4
Plasma triglycerides (mg/dl)	64 ± 25	87 ± 46
Plasma cholesterol (mg/ml)	45 ± 13	39 ± 10

Data are expressed as mean ± SD (n=4-6 in each group).

Mice

Previously, we established a conditional, inducible *Gata4* mutation model[14] that consists of mice homozygous for the *Gata4*^{flox} allele[20] and heterozygous for a transgene expressing the tamoxifen-activated CRE recombinase under the control of the villin promoter.[18] In the *Gata4*^{flox} allele, exon 2 is flanked by LoxP sites, and CRE-mediated recombination of the inserted LoxP sites results in excision of exon 2, and the synthesis of a truncated form of GATA4 devoid of its activation domains. This causes GATA4 activity in the small intestine to be reduced but not eliminated (Model 1, *G4Δex2*, Supplementary Figure 1).[14] To inducibly delete *Gata4* in the mature small intestine, we established a new model (Model 2, *G4ap*, Supplementary Figure 1) using a previously published modification of the *Gata4* allele.[16] In Model 2, the coding region for the endogenous *Gata4* gene was replaced by a cassette encompassing a *Gata4* cDNA with an amino terminal epitope tag derived from the influenza HA protein (*HA-Gata4*), which is followed by a Stop repressor sequence and a placental alkaline phosphatase (PLAP) expression cassette. LoxP sites were inserted before the HA-tagged *Gata4* cDNA and after the Stop repressor cassette. CRE-mediated recombination results in excision of *HA-Gata4* and the Stop repressor cassette, and synthesis of PLAP under the control of the endogenous *Gata4* promoter. In both models, conditional recombination was targeted to the small intestinal epithelium by the Villin*Cre*ER^{T2} transgene.[14, 17, 18] To induce recombination, and also to control for potential tamoxifen effects, all animals were treated with one intraperitoneal injection of tamoxifen (Sigma-Aldrich, St. Louis, MO) (100µl, 10mg/ml) per day for 5 consecutive days as described.[14, 17] Tissue was collected 14-17 days after the last tamoxifen injection. All mice were previously backcrossed into the C57BL/6 genetic background and only adult (6-12 wk of age) males were used for study. Mice were fed ad libitum standard rodent chow containing approximately 5% w/w fiber and 60% carbohydrate, 28% protein, and 12% fat (% of calories), unless indicated otherwise. Genotypes were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) using previously validated primers (Supplementary Figure 2).[14, 16, 18-20] The genotypes for all control and test mice are indicated in Supplementary Table 1. Approval was obtained from the Institutional Animal Care and Use Committee.

RNA isolation and reverse transcriptase polymerase chain reaction

Mice were dissected and RNA was isolated from liver and small intestine using the RNeasy kit (Qiagen) as described previously.[14] Intestinal tissue samples 1 to 5 (approximately 1.0 cm in length) are indicated as the gray boxes in Figure 1A. The tissue samples were taken from the most proximal region adjacent to the pylorus (segment 1), the 25% mark (segment 2), the geometric center (segment 3, also indicated as jejunum), the 75% mark (segment 4), and the most distal region adjacent to the ileocecal junction (segment 5, also indicated as ileum).

Messenger RNA (mRNA) abundances were determined by semi-quantitative and real-time RT-PCR[14] using validated primer pairs (Supplementary Figure 2 and 3) designed with Beacon Designer™ software (PREMIER Biosoft International, Palo Alto, CA). Semi-quantitative RT-PCR was terminated in the linear range of amplification, and real-time RT-PCR was carried out using

an iCycler and iQ SYBR GreenSupermix (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase mRNA abundance was measured for each sample and used to normalize the data.

Protein extracts and immunoblotting.
After intestinal samples were obtained for RNA isolation, brush border membrane vesicles (BBMV) were prepared as described by Kessler et al[21] on the remaining four quarters of intestine (Figure 1A). Intestinal segments were placed on a glass plate on a bed of wet ice, rinsed in ice cold PBS containing protease inhibitors, and cut longitudinally along the antimesenteric border. Epithelial cells were then collected by scraping the mucosa with a glass microscope slide. The scrapings were suspended in 20 mL of buffer A (50 mmol/L mannitol, 2 mmol/L Tris, pH 7.2, 50 μg/mL benzamidine) and homogenized on ice with 10 strokes using a ground glass homogenizer. Solid $CaCl_2$ was added to a final concentration of 10 mmol/L, incubated on ice for 15 min, and centrifuged (3000 relative centrifugal force (rcf) for 10 min at 4°C). The supernatant was centrifuged again (24330 rcf for 20 min at 4°C) and the pellet was resuspended in 5 mL of buffer B (300 mmol/lL mannitol, 10 mmol/L Tris, pH 7.2, 50 ug/ml benzamidine). The homogenate was again centrifuged (24330 rcf for 30 min at 4°C), the pellet was resuspended in 200 μL of buffer B, and the concentration of proteins was quantified as described.[53] The proteins were stored at -80°C until used for immunoblotting.

Western analysis was conducted as described previously[22] using 30 μg of BBMV. Primary antibodies included rabbit anti-ASBT[23] (1:3750) and mouse anti-β-actin (1:4000, Sigma). After probing with rabbit anti-ASBT, membranes were stripped and re-probed using mouse anti-β-actin (1:4000, Sigma). Horseradish peroxidase-linked secondary antibodies and chemiluminescence solution (Pierce West Femto Kit) were used to visualize the signals.

Bile acid measurements
Bile acid transport in everted gut sacs. The small intestine was divided into quartiles I – IV (depicted as white boxes in Figure 1A), and the mucosal-to-serosal bile acid transport was measured using everted gut sacs as previously described.[24] After removal of adherent fat, each segment was weighed, gently everted, filled with oxygenated Krebs Ringer Buffer (KRB), and closed using suture and weighed again. The closed sacs were incubated at 37°C for 30 min in oxygenated KRB containing 25 μM [^{3}H]taurocholate (final specific activity = 55 mCi/mmol; Perkin-Elmer) and inulin carboxylic acid (2 mCi/mmol; Perkin-Elmer). After incubation, the sacs were removed and weighed, and the serosal fluid was recovered. The empty sacs were solubilized in sodium hydroxide, and aliquots of mucosal fluid, serosal fluid, and gut sac tissue extract were taken for radioactivity measurements. The amount of inulin [^{14}C]carboxylic acid associated with the sac or serosal fluid was similar for the different segments and genotypes, and was used to correct the sac-associated [^{3}H]taurocholate data for leakage and paracellular movement.

Bile acid abundance and composition in stools and tissues. Bile acid abundance and composition were determined in fecal material and in tissues. For total fecal bile acid excretion, mice were

weighed, individually housed in wire-bottom cages for 72 h beginning 14 days after the completion of tamoxifen treatment, and the feces were collected. Bile acids were extracted from the fecal samples as described[54] and aliquots of the final methanol extract were taken to measure the radioactivity of the [^{14}C]cholic acid recovery control and to measure the bile acid content using an enzymatic assay.[55] For measurement of bile acid concentrations in segmental luminal contents, the small intestine was removed and divided into four quarters, the segments were cut longitudinally, and the luminal content of each segment was collected as described.[26] Briefly, an internal standard (~50,000 dpm of [^{14}C]cholic acid) is added to the luminal contents. The samples are air-dried and extracted twice with methanol. Aliquots of the methanol extract are taken to measure the radioactivity and to measure the bile acid content using an enzymatic assay.[55] Tissue-associated bile acids in each of the four quarters of small intestine were also measured. Briefly, an internal standard (~200,000 dpm of [^{14}C]cholic acid) is added to the tissue. The tissue is then minced and extracted twice with ethanol at 65° C. The ethanol extracts are pooled, dried, and resuspended in methanol. Aliquots of the methanol extract are taken to measure the radioactivity and to measure the bile acid content using an enzymatic assay.[55]

For bile acid pool measurements, mice were fasted for 5 h and the liver, gall bladder and small intestine were collected and bile acid extracted as described.[56] Briefly, approximately 2.7 nmoles of nor-deoxycholic acid is added to the tissue as an internal standard. The tissue is homogenized in ethanol, and extracted twice with ethanol at 65°C. The ethanol extracts are dried, resuspended in water and applied to a C18 reverse phase column. The bile acids are eluted with methanol and analyzed using high-performance liquid chomatography and an evaporative light scatter detector (Alltech ELSD 800).[57] Bile acids were identified and quantified by comparison to known amounts of authentic standards purchased from Steraloids (Newport, RI). In selected experiments, bile acid composition was also determined by high performance liquid chromatography for the luminal contents. The bile acid hydrophobicity index was calculated according to Heuman.[27]

Surgery

Ten to twelve week old male mice were transferred to a liquid rodent diet (Bio-serve Inc, Frenchtown, NJ) two days prior to surgery. Mice were weighed and sham or ICR surgery was performed as previously described[28] (Supplementary Figure 4). In mice that underwent ICR, ~10 cm of ileum proximal to the ileocecal junction and cecum were removed, and the colon was anastomosed to the remaining small intestine. *Asbt* mRNA abundance in the most proximal one-cm of the resected small intestine was low or undetectable, indicating that all of the native *Asbt*-expressing tissue was removed. In mice that underwent sham operations, transection and anastomosis occurred ~10 cm proximal to the ileocecal junction. Mice were given 2 ml intraperitoneal zosyn (100 mg/kg, Wyeth Pharmaceuticals, Philadelphia, PA) in phosphate-buffered saline and the abdomen was closed. The mice were incubated at 27°C for 2-4 h after surgery, and then transferred to their normal housing, with access to liquid diet. After a week of recovery, the mice were transitioned to solid food. Three weeks after surgery, mice were treated with tamoxifen and tissue was collected

as described above. All mice treated with tamoxifen were fully recovered from surgery as indicated by normal activity, weight gain to pre-surgical body weights, and formed stools. The overall mortality rate was 59%; virtually all mice that did not recover from surgery died of obstruction.

Statistical analyses

Data are expressed as mean ± SD. Statistically significant differences were determined by the two-tailed Student's *t* test or analysis of variance followed by the Tukey-Kramer multiple comparison test. Differences were considered statistically significant at $P<0.05$.

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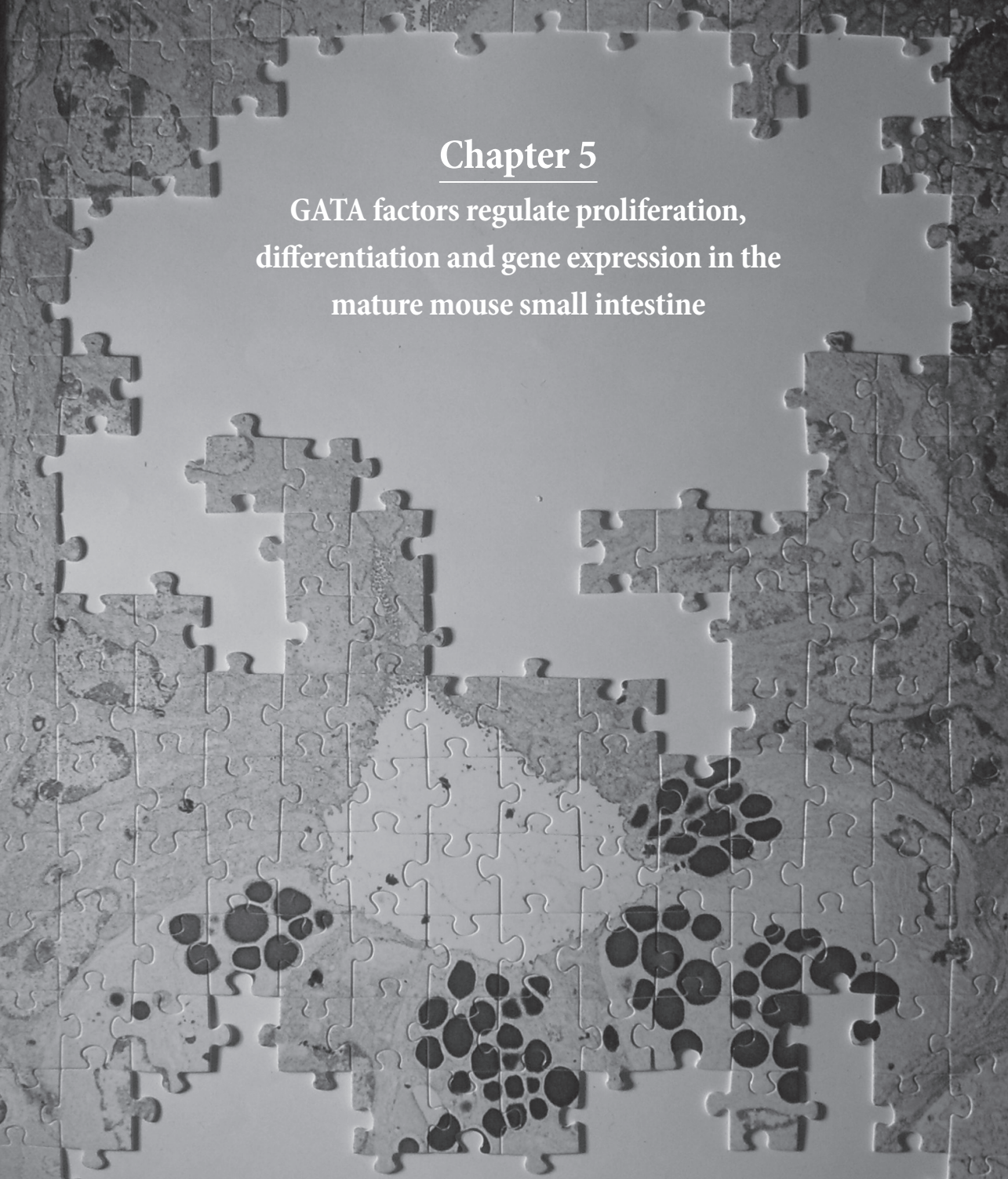
Chapter 5

GATA factors regulate proliferation, differentiation and gene expression in the mature mouse small intestine



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ABSTRACT

Background & Aims: GATA transcription factors play key roles in proliferation, differentiation and gene regulation in multiple organs. In small intestine, GATA4 is expressed in the proximal 85% where it regulates the expression of specific absorptive enterocyte genes. GATA6 is co-expressed with GATA4 but is also expressed in the ileum; its function in the mature small intestine is unknown. **Methods:** To determine the function of GATA6 in the small intestine, *Gata6* or *Gata6* and *Gata4* were inducibly deleted specifically in the intestine of adult mice. **Results:** In ileum, deletion of *Gata6* resulted in a decrease in proliferation, an increase in goblet-like cells in crypts, a loss of Paneth cells, a reduction in enteroendocrine cells, and altered expression of specific absorptive enterocyte genes. In jejunum, deletion of *Gata6* resulted in an increase in Paneth cells, possibly as a compensatory response to the loss of Paneth cells in the ileum. Deletion of both *Gata6* and *Gata4* resulted in a jejunal phenotype that was similar, but not identical, to that in the ileum after *Gata6* deletion alone, demonstrating that GATA4 is redundant for many GATA6 functions. **Conclusion:** GATA factors maintain the cellular dynamics in the small intestinal epithelium by regulating proliferation, differentiation, lineage commitment, and absorptive enterocyte gene expression.

INTRODUCTION

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 ultimately give 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, necessary for maintaining a dynamic mucosal defensive barrier, are more numerous in distal small intestine where bacterial challenge is greater, 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] The mechanisms that guide the continuous renewal and precise differentiation and distribution of cell types of the small intestinal epithelium are not yet fully understood.

GATA proteins are highly conserved transcription factors that regulate proliferation, differentiation, and gene expression in multiple organs.[2] GATA4 is expressed in the proximal 85% of adult small intestinal epithelium but is absent from distal ileum,[3, 4] whereas GATA6 is expressed throughout the small intestine, including distal ileum. To define the function of GATA4 in adult small intestine, we previously established a conditional, inducible inactivation model in which GATA4 expression is replaced by GATA4ΔEx2, a functionally inactive form that continues to bind DNA *in vitro* and thus has potential dominant-negative activity by competing with other GATA factors for DNA binding sites. Expression of GATA4ΔEx2 in place of GATA4 in mouse small intestine resulted in a jejunum-to-ileum transformation in absorptive enterocyte gene expression as well as an expansion of goblet cells and a redistribution of enteroendocrine cell types.[3] To determine which of these changes are due to loss of GATA4, and which are due to a dominant-negative effect by GATA4ΔEx2, we also established a conditional, inducible *Gata4* deletion model.[5] In this model, the jejuno-ileal changes in absorptive enterocyte gene expression were preserved,[5] but secretory cell numbers or distribution were not altered (unpublished observation). These findings suggest that the changes in absorptive enterocyte gene expression were due to the loss of GATA4 and that GATA4ΔEx2 has a dominant-negative effect on secretory cell differentiation. This is consistent with the hypothesis that GATA4 specifically regulates the jejuno-ileal differences in absorptive enterocyte gene expression, but is redundant, likely with other GATA factors, for secretory cell differentiation. Because GATA6 is also expressed in the small intestine, we hypothesized that GATA4 and/or GATA6 are necessary for secretory cell differentiation, and possibly other functions, in this organ. To test this hypothesis, we defined the structure, cell lin-

eage characteristics, and gene expression in the jejunum and ileum of single *Gata6* and double *Gata4/Gata6* conditional, inducible knockout mice.

MATERIALS AND METHODS

Mice

Previously established and confirmed *Gata6*$^{loxP/loxP}$, *Gata4*$^{flap/flap}$ and transgenic Villin*Cre*ER^{T2} mice[3, 5-7] 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*Cre*ER^{T2}-positive (*G6del*), *Gata6*$^{loxP/loxP}$, *Gata4*$^{flap/flap}$, Villin*Cre*ER^{T2}-positive (*G6G4del*), and *Gata6*$^{loxP/loxP}$, Villin*Cre*ER^{T2}-negative or *Gata6*$^{Wt/Wt}$, Villin*Cre*ER^{T2}-positive (*Control*) mice, 6-8 wk of age, were treated with tamoxifen as described.[3, 5, 8] Tissue was collected 28 days after treatment, unless otherwise indicated. Approval was obtained from the Institutional Animal Care and Use Committee.

Tissue isolation

Mice were dissected as previously described.[3] Samples of ileum were taken from distal small intestine adjacent to the ileocecal valve whereas samples of jejunum were taken from the geometric center of the small intestine. In selected mice, bromodeoxyuridine (BrdU) (0.1 ml of 10 mg/ml) was injected one hour prior to dissection.

Sectioning, staining, immunohistochemistry, and electron microscopy

Intestinal segments were fixed as previously described.[3] Primary and secondary antibodies used for immunostaining are listed in supplementary expanded Materials and Methods. Sections were counterstained with alcian blue, methyl green, and/or hematoxylin.

For electron microscopy (EM), intestinal segments were fixed in 1.25% gluteraldehyde, 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 epithelial cells. The total number of alcian blue- or Ki67-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. For all determinations (blinded and conducted on a minimum of 5 animals per group), a minimum of six villi or six crypts per slide were analyzed.

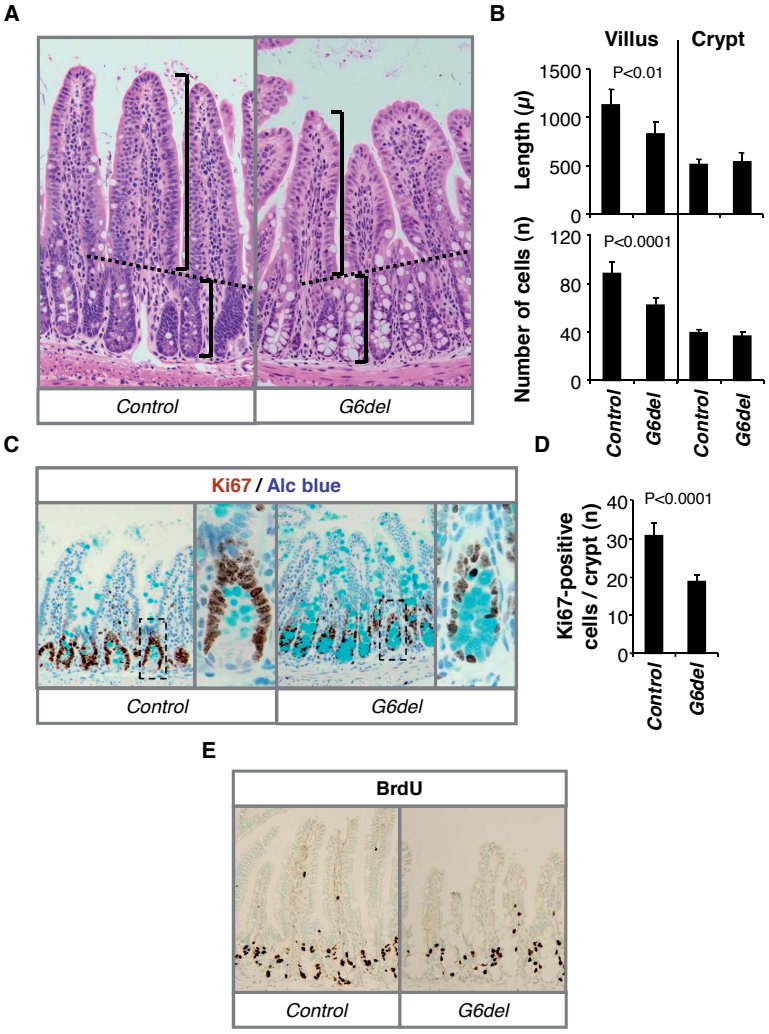

Figure 1. Intestinal *Gata6* deletion results in a reduction in proliferation in the mature ileum. (A) From hematoxylin and eosin stained slides, crypt-villus junctions were established (dotted line) and villus height and crypt depth (brackets), as well as villus and crypt cell number, were determined. (B) Villus height and cell number were decreased in *G6del* as compared to *Control* ileum. (C) Immunostaining for Ki67 revealed that (D) the number of positive cells was decreased after *Gata6* deletion. (E) Immunohistochemistry demonstrated a decrease in the number of bromodeoxyuridine (BrdU)-positive cells after *Gata6* deletion.

RNA isolation and gene expression analysis

RNA was isolated from 0.5 to 1.0 cm intestinal segments as described previously.[3, 5, 8] Gene expression was determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) as previously described[3, 5, 8] using validated primer pairs (Supplementary Figure 1), and ileal messenger RNA (mRNA) was analyzed by whole-genome gene expression analysis using the Affyme-

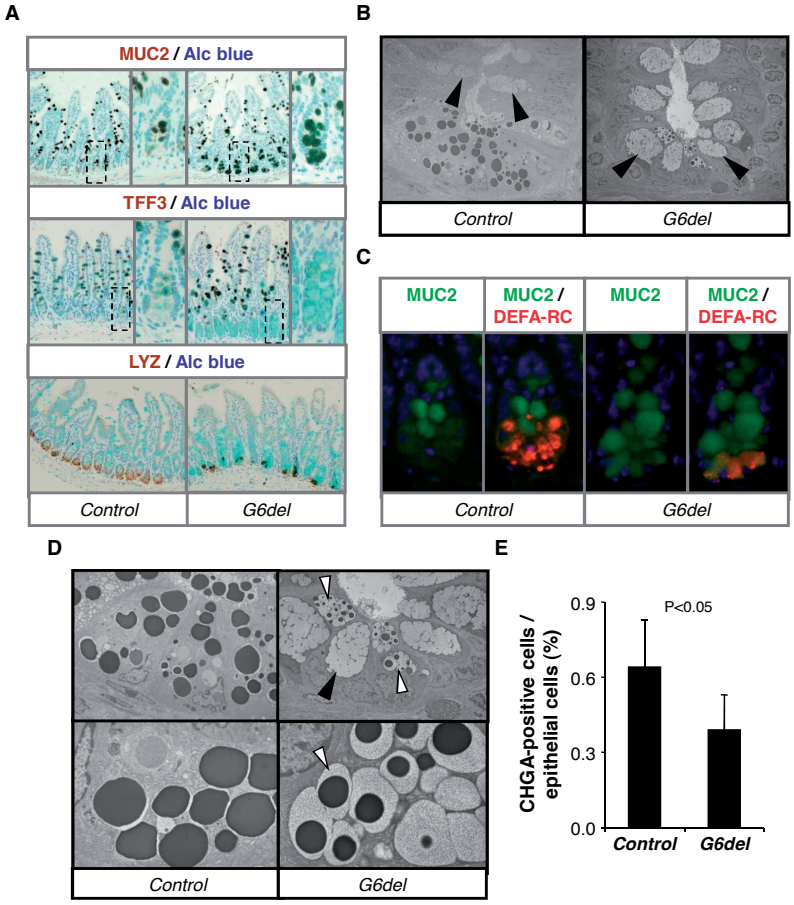

Figure 2. Intestinal *Gata6* deletion results in an increase in goblet-like cells and a decrease in Paneth and enteroendocrine cells. (A) Immunohistochemistry showed that goblet-like cells in crypts of *G6del* ileum were positive for alcian blue and mucin 2 (MUC2) and negative for trefoil factor 3 (TFF3), and revealed fewer lysozyme (LYZ)-positive cells in crypts. (B) Electron microscopy revealed characteristic Paneth cells at the base of crypts in *Control* ileum that are replaced by cells with structural goblet-like features at the base of crypts in *G6del* ileum. Arrowheads indicate cells with goblet characteristics. (C) Immunostaining revealed that a subpopulation of the goblet-like cells in the crypt of *G6del* ileum co-express mucin 2 (MUC2) and alpha-defensin related sequence (DEFA-RS). (D) Electron microscopy revealed that typical Paneth granules in *Control* ileum are not found in *G6del* ileum, and that a subpopulation of crypt cells in *G6del* ileum contain granules with both Paneth and goblet characteristics (white arrowheads). (E) Cell counting revealed a decrease in the number of chromogranin A (CHGA)-positive cells per total epithelial cells after *Gata6* deletion.

trix Mouse Gene 1.0 ST array by the molecular genetics core facility at Children's Hospital Boston (see supplementary expanded Materials and Methods).

Statistical analyses

Data are expressed as mean ± SD. Statistically significant differences were determined by the two-tailed Student's *t* test or analysis of variance followed by the Tukey-Kramer multiple comparison test. Differences were considered statistically significant at $P<0.05$.

Expanded Material and Methods

See supplementary files.

RESULTS

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

Consistent with data from humans, 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).[9] To determine the function of GATA6 in this tissue, an inducible, intestine-specific *Gata6* deletion model (*G6del*) was established (Supplementary Figure 3) using mice carrying a previously validated floxed *Gata6* allele[7] and Villin*Cre*ER^{T2} transgene.[6] Mice sacrificed up to 6 weeks after *Gata6* deletion exhibited normal growth and activity. Initial studies focused on distal ileum, where GATA6 is normally expressed, but GATA4 is not.

In the ileum of *G6del* mice, villi were 27% shorter and contained 29% fewer cells as compared to *Control* ileum, whereas the depth and cell number of crypts remained unchanged (Figure 1A and B). Cleaved caspase 3 immunostaining was not different between *G6del* and *Control* ileum (data not shown) but a reduction in Ki67-positive (Figure 1C and D) and BrdU-positive cells (Figure 1E) was found, suggesting that the decrease in villus height and cell number is due to a reduced rate of crypt cell proliferation and not increased apoptosis. Networks constructed from microarray data revealed a general increase in the expression of targets of the tumor suppressor transcription factor p53, and a general decrease in the expression of targets of the myelocytomatosis oncogene (c-MYC) (Supplementary Figure 4), supporting a change toward a less proliferative state after *Gata6* deletion.

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

Alcian blue staining was increased in crypts from the ileum of *G6del* mice (Figure 1C). There was no change in the number of alcian blue positive cells on villi, but a 1.9-fold increase in positive cells in crypts ($P<0.0001$). The alcian blue-positive crypt cells also expressed mucin 2 (MUC2), an early marker of differentiating goblet cells, but not trefoil factor 3 (TFF3), a late marker of differentiating goblet cells (Figure 2A). In agreement, *Muc2* mRNA abundance was increased

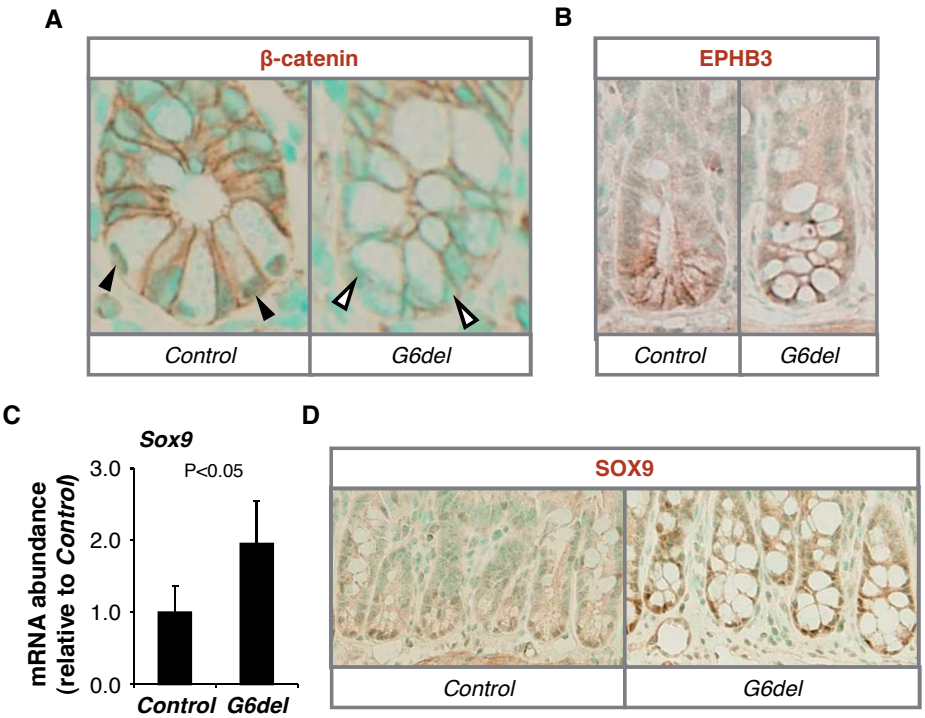

Figure 3. Intestinal *Gata6* deletion results in a Paneth cell-specific decrease in Wnt signaling. Immunostaining revealed (A) a decrease in nulcear β-catenin in cells at the base of the crypts after *Gata6* deletion and (B) that eph receptor B3 (EPHB3) is expressed in the goblet-like cells in the crypts. (C) qRT-PCR showed an increase in SRY-box containing gene 9 (*Sox9*) mRNA in *G6del* as compared to *Control* ileum (n=5 in each group). (D) Immunostaining revealed an increase in SOX9 staining in the crypt compartment after *Gata6* deletion.

1.6-fold (P<0.05), whereas that of *Tff3* remained unchanged (Supplementary Figure 5A). These data suggest that the goblet cell differentiation and migration programs remains intact, but that goblet-like cells accumulate in the crypts after *Gata6* deletion.

Immunostaining for the Paneth cell marker lysozyme (LYZ) revealed a decrease in crypt staining and an overlap with alcian blue-positive cells in *G6del* ileum (Figure 2A, bottom panels). *Lyz* mRNA abundance was reduced by 92% (P<0.001, Supplementary Figure 5A), coinciding with a 79% reduction in the number of LYZ-positive cells (P<0.0001). The decline in *Lyz* mRNA occurred more than 2 weeks after tamoxifen (Supplementary Figure 5B), coinciding with a decrease in typical Paneth cells and an increase in goblet-like cells in crypts (Supplementary Figure 5C). Structural analyses by transmission EM demonstrated that Paneth cells (with typical electron dense granules that normally localize to the base of crypts in *Control* ileum) were replaced by cells containing typical mucin granules in the *G6del* ileum (Figure 2B). Gain of goblet-like cells and loss of Paneth cells was supported by microarray analysis, which showed a general increase in the

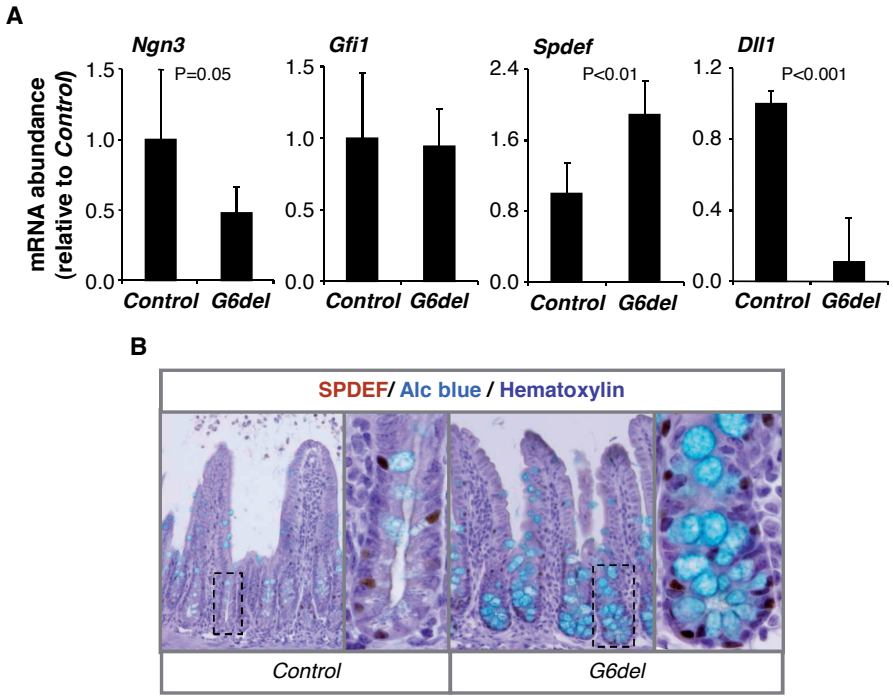

Figure 4. Intestinal *Gata6* deletion results in alterations in Notch signaling targets. (A) qRT-PCR showed an increase in SAM pointed domain containing ets transcription factor (*Spdef*) and decreases in neurogenin 3 (*Ngn3*) and delta-like 1 (*Dll1*) mRNA abundance in *G6del* as compared to *Control* ileum. (B) Immunohistochemistry revealed an increase in the intensity of SPDEF-expressing cells in the ileal crypts after *Gata6* deletion.

expression of goblet marker transcripts, and a general decline in the expression of Paneth markers transcripts (Supplementary Figure 6A). 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 arrowheads, Figure 2D). These granules were clearly different from 'mixed' granules in nascent intermediate or granular goblet cells described previously[10] and are likely the granules that co-express goblet and Paneth markers.

Microarray analysis also revealed a general decline in transcript abundance for enteroendocrine markers (Supplemental Figure 6A), which we have confirmed by qRT-PCR for *Chga*, glucagon (*Gcg*) and peptide YY (*Pyy*) (Supplemental Figure 7A). The percentage of CHGA-positive enteroendocrine cells was reduced in *G6del* ileum (Figure 2E), indicating that the general decline in marker transcripts is due to a reduction in the number of enteroendocrine cells.

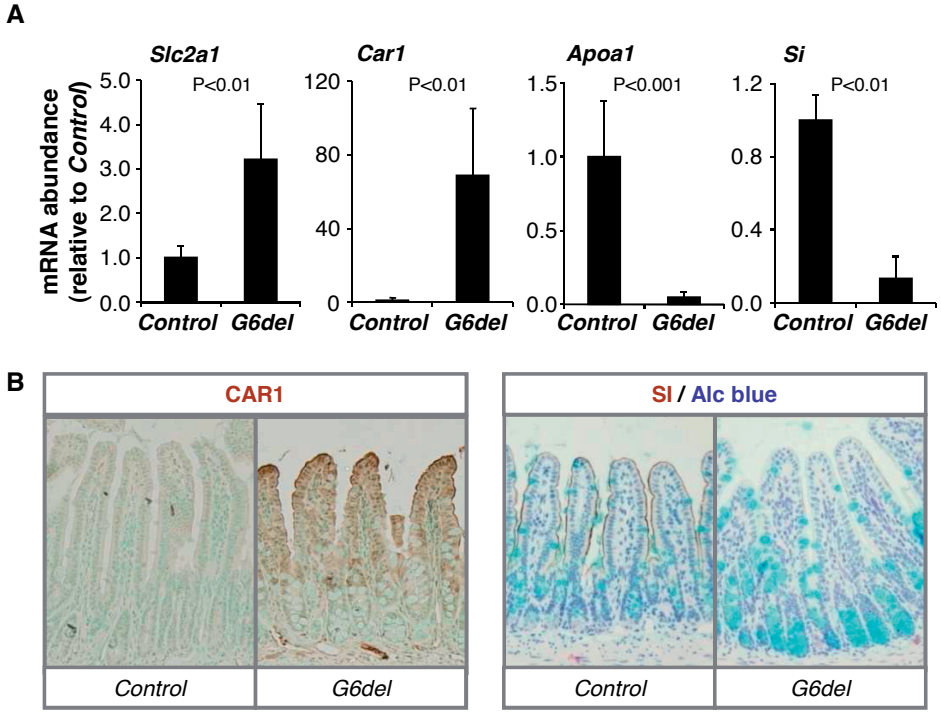

Figure 5. Intestinal *Gata6* deletion results in alterations in the expression of specific absorptive enterocyte genes. (A) qRT-PCR showed increases in solute carrier family 2, member 1 (*Slc2a1*) and carbonic anhydrase 1 (*Car1*), and decreases in apolipoprotein A-I (*Apoa1*) and sucrase isomaltase (*Si*) mRNA abundance in *G6del* as compared to *Control* ileum. (B) Immunohistochemistry revealed an increase in CAR1 and a decrease in SI staining in absorptive enterocytes after *Gata6* deletion.

Intestinal *Gata6* deletion results in a Paneth cell-specific decrease in Wnt signaling in ileum.

Since the interplay between Wnt and Notch signaling regulates intestinal proliferation and differentiation,[11] we investigated these pathways in the *G6del* mice. Nuclear β-catenin, the hallmark of active Wnt signaling within cells,[12] 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 Wnt target alpha-defensin 1 (*Defa1*) was also decreased (Supplementary Figure 7B), suggesting that Paneth-specific Wnt signaling is decreased. Reduced Wnt signaling in *G6del* ileum could be due to a decreased expression of the Paneth-specific Wnt receptor, frizzled 5 (*Fzd5*), which is down-regulated in the *G6del* ileum (Supplementary Figure 7B) perhaps as a consequence of a loss of Paneth cells. In contrast to the reduced transcript abundance of Paneth-specific Wnt targets, other Wnt targets were either unchanged (*Ephb2*, *c-Myc*) or up-regulated (*Cd44*) (Supplementary Figure 7C), indicating that Wnt signaling is not sig-

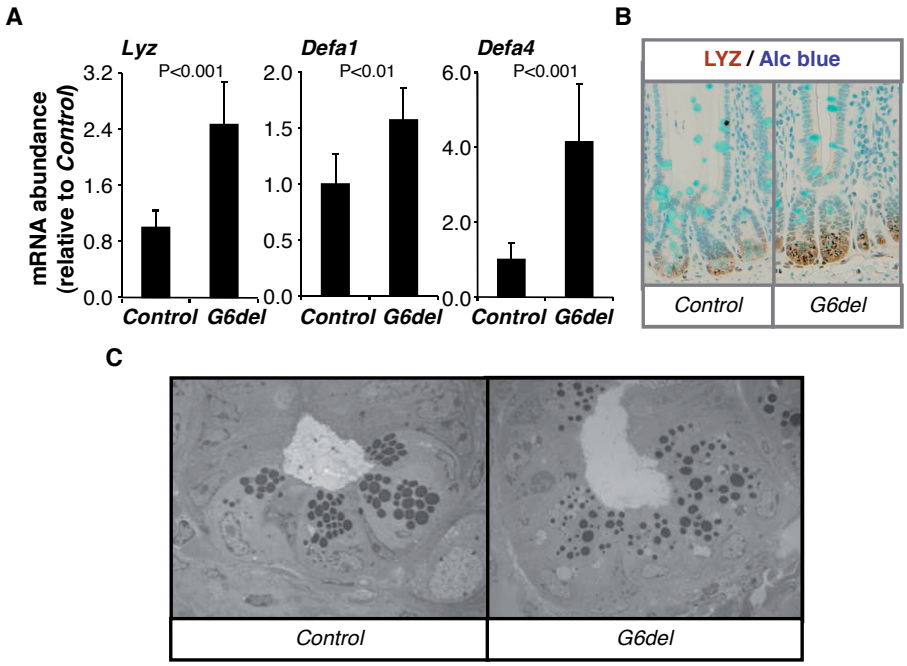

Figure 6. Intestinal *Gata6* deletion results in an increase in atypical Paneth cells in the jejunum. (A) qRT-PCR showed increases in lysozyme *(Lyz),* alpha-defensin 1 (*Defa1*) and alpha-defensin 4 (*Defa4*) mRNA abundance after *Gata6* deletion. (B) Immunohistochemistry revealed an increased intensity and a more widespread pattern of LYZ staining in jejunal crypts after *Gata6* deletion. (C) Electron microscopy revealed Paneth granules that were more variable in size and more widely dispersed in the cell than normal.

nificantly compromised outside of the Paneth cell compartment. The eph receptor B3 (EPHB3), a Wnt target expressed in the crypt base region and necessary for Paneth cell localization,[13] was expressed in the goblet-like cells at the base of crypts (Figure 3B) likely explaining their targeting to this position. The mRNA for the transcription factor, SRY-box containing gene 9 (SOX9), a Wnt target also expressed in Paneth progenitors and required for Paneth cell differentiation,[14, 15] was up-regulated (Figure 3C), and SOX9 immunostaining exhibited a more intense signal in the goblet-like cells in the crypts of *G6del* vs. *Control* ileum (Figure 3D), indicating that the decline in Paneth cells in *G6del* ileum is not due to a decrease in SOX9 expression. The up-regulation of SOX9 could be a compensatory response to the loss of Paneth cells.

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

Notch signaling maintains the undifferentiated state of the crypt progenitors, and is also required for commitment to the absorptive enterocyte cell lineage by activating the expression of hairy and enhancer of split 1 (HES1).[16-18] Progenitor cells that escape Notch signaling and HES1 acti-

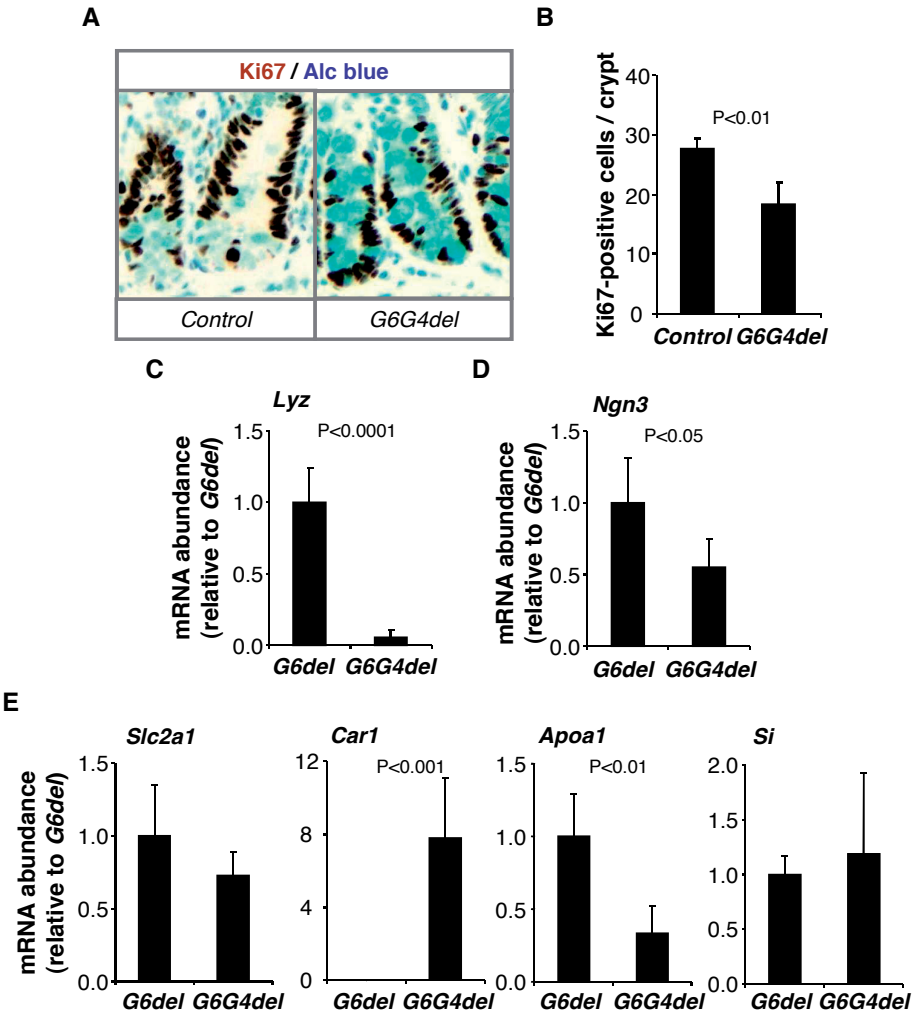

Figure 7. GATA4 is redundant for most GATA6 functions in the mature jejunum. (A) Sections stained for Ki67 and alcian blue (Alc blue) revealed (B) a decrease in the number of Ki67-positive cells per crypt in *G6G4del* as compared to *Control* jejunum. (C, D) qRT-PCR showed decreases in (C) lysozyme *(Lyz)* and (D) neurogenin 3 *(Ngn3)* mRNA abundances in *G6G4del* as compared to *G6del*. (E) qRT-PCR showed an increase in carbonic anhydrase (*Car1*) and a decrease in apolipoprotein A-I (*Apoa1*) mRNA abundance in *G6G4del* as compared to *G6del* jejunum.

vation express atonal homolog 1 (ATOH1) that selects the secretory cells.[19] Microarray analysis on *G6del* ileum did not identify alterations in *Notch1* or *Hes1* transcript abundance, but revealed an increase in *Atoh1* mRNA (Supplementary Figure 6A), consistent with an expansion of secretory progenitors and/or mucous cells.[19] Secretory progenitors that express neurogenin 3 (NGN3) become enteroendocrine cells, whereas growth factor independent 1 (GFI1) promotes goblet/

Paneth lineage differentiation at the expense of enteroendocrine cells.[20] In *G6del* ileum, *Ngn3* mRNA abundance was down-regulated, consistent with a decrease in enteroendocrine cell lineage commitment, whereas that of *Gfi1* mRNA remained unchanged (Figure 4A).

Previously, conditional over-expression of the gene encoding SAM pointed domain containing ets transcription factor (SPDEF), a downstream target of GFI1, was shown to cause cell cycle arrest and an expansion of the goblet cell lineage at the expense of the other lineages in the small intestine.[21] This is very similar to the outcome in our intestinal *Gata6* deletion model. Conditional deletion of *Spdef* impairs goblet and Paneth cell maturation, and causes accumulation of secretory progenitors, i.e. more crypt cells expressing the Notch ligand delta-like 1 (DLL1).[22] DLL1 inhibits secretory cell differentiation by activating Notch signaling in adjacent cells, thereby generating a homeostatic mixture of absorptive and secretory cells.[22, 23] Inhibition of Delta-Notch signaling causes overproduction of secretory cells due to loss of lateral inhibition and a subsequent default to the secretory lineage. In *G6del* mice, *Spdef* mRNA abundance was up-regulated, and *Dll1* mRNA abundance was down-regulated (Figure 4A). The up-regulation of SPDEF was localized to crypt cells (Figure 4B). These data suggest that GATA6 modulates the Notch signaling pathway and secretory cell differentiation by repressing *Spdef* and activating *Dll1* expression. To define the placement of GATA6 within this pathway, we obtained RNA from *Gfi1-/-*[20] and *SpdefKO*[22] intestine, and from conditional transgenic *Spdef* over-expressing crypts (*SpdefTG*).[21] In all models, *Gata6* mRNA levels were not changed (data not shown) suggesting that *Gata6* is not a downstream target of either GFI1 or SPDEF.

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

GATA6 is expressed in absorptive enterocytes on villi (Supplementary Figure 2) and thus may regulate gene expression in this lineage. In *G6del* ileum, genes normally expressed at low or undetectable levels in small intestine but highly expressed in colon were up-regulated (e.g. carbonic anhydrase (*Car*) 1 and 2, and claudin 8), while genes normally expressed in absorptive enterocytes were down-regulated (e.g. genes for proteins involved in lipid metabolism, including apolipoprotein C-III, apolipoprotein A-I (*Apoa1*), and fatty acid binding protein 6) (Supplementary Figure 6B). In agreement with these observations, functional analyses of the microarray data showed that gene expression changes that occur following conditional *Gata6* deletion are likely to affect lipid metabolism. Changes in transcript abundance were confirmed by qRT-PCR for selected genes (up: solute carrier family 2, member 1 (*Slc2a1*), *Car1;* down: *Apoa1*, sucrase isolmaltase (*Si*)*;* Figure 5A). At the protein level, the changes in CAR1 and SI were localized to absorptive enterocytes on villi (Figure 5B).

Intestinal *Gata6* deletion results in an increase in aberrant Paneth cells in the jejunum.

All of the analyses described above were performed on ileum where GATA6 is normally expressed, but GATA4 is not.[3, 4] In jejunum, where GATA4 and GATA6 are normally co-expressed, *Gata6* deletion did not alter the number of Ki67-positive proliferating, alcian blue-positive goblet, or CHGA-positive enteroendocrine cells, nor did it alter the mRNA levels of the absorptive enterocyte genes *Slc2a1*, *Car1, Apoa1* and *Si*, that were affected in the ileum (data not shown). *Gata6* deletion also did not alter the expression of *Gata4* or its targets lactase-phlorizin hydrolase (*Lph*) and apical sodium-dependent bile acid transporter (*Asbt*)[3, 5] (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), as well as the immunostaining intensity of LYZ (Figure 6B), all increased in *G6del* jejunum as compared to *Control* jejunum. 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 while GATA6 may be required for proper Paneth cell granule formation in the jejunum, it is dispensable for proliferation, most aspects of Paneth cell differentiation, enteroendocrine commitment and the expression of specific absorptive enterocyte genes.

GATA4 is redundant for most but not all GATA6 functions in the mature jejunum.

Deletion of both *Gata6* and *Gata4* in the jejunum (confirmed by qRT-PCR and immunostaining; data not shown) resulted in a jejunum phenotype that was similar to that found in the *G6del* ileum: fewer Ki67-positive proliferating cells (Figure 7A and B); more alcian blue- (Figure 7A) and MUC2-positive, goblet-like cells in crypts (data not shown); lower mRNA levels of the Paneth cell markers *Lyz* (Figure 7C) and *Defa1* (decrease of 94%, $P<0.001$); 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); and a decrease in the mRNA abundance of the enteroendocrine cell markers *Ngn3* (Figure 7D) and *Pyy* (decrease of 80%, $P<0.05$). The mRNA level for the absorptive enterocyte marker *Car1* was increased and for *Apoa1* was decreased, whereas *Slc2a1* and *Si* mRNA abundances were unchanged (Figure 7E). These data indicate that the lack of effect of GATA6 deletion on proliferation, Paneth cell differentiation, enteroendocrine lineage allocation and absorptive enterocyte gene expression in the jejunum is due to functional redundancy with GATA4.

DISCUSSION

Cellular proliferation in intestinal crypts is necessary for the maintenance of crypt and villus integrity, and for the continuous renewal of the intestinal epithelium. Here, we show that conditional deletion of *Gata6* results in a decrease in proliferation in crypts and a corresponding

reduction in villus height and epithelial cell number in ileum (Figure 1). Conditional deletion of both *Gata6* and *Gata4* causes a similar decrease in proliferation in jejunum (Figure 7A and B), indicating that GATA6 or GATA4 is required to maintain normal crypt cell proliferation and villus epithelial renewal. In bronchiolar lung epithelium, *Gata6* deletion results in an increase in cell proliferation,[24] and in vascular smooth muscle cells, GATA6 induces the expression of the cyclin-dependent kinase inhibitor, $p21^{Cip1}$ resulting in cell cycle arrest.[25] These data suggest that GATA factors can both promote and inhibit cellular proliferation depending on the organ or cell type in which they are expressed.

Paneth cell loss and goblet-like cell accumulation at the crypt base where Paneth cells normally reside did not occur until at least 2 weeks after the 5-day tamoxifen treatment (Supplementary Figure 5). This is consistent with the slower turnover rate of Paneth cells and indicates a defect in the Paneth cell differentiation program. The goblet-like cells that accumulate in crypts display a nearly complete spectrum of goblet cell characteristics (Figure 2A and B), but also express genes that promote Paneth cell differentiation and their crypt localization, including SOX9 (Figure 2D) and EPHB3 (Figure 2B). A small percentage of cells also have mixed goblet-Paneth features (Figure 2C and D). We believe that the goblet-like cells that accumulate in the crypts of *G6del* mice are committed Paneth cells that, in the absence of GATA6, fail to differentiate appropriately and by default continue to differentiate to a goblet-like cell type.

The current model of intestinal epithelial differentiation proposes that Wnt signaling drives the production of a pool of multipotent progenitors that utilize Notch signaling to select between absorptive and secretory progenitors.[17] Our data suggest that intestinal *Gata6* deletion results in a Paneth cell-specific decrease in Wnt signaling that likely results from the loss of differentiated Paneth cells. Our data also indicate that overall Wnt signaling is not compromised, as shown by the expression/over-expression of the Wnt target genes EPHB3, SOX9, and SPDEF in the goblet-like cells at the crypt base. We believe that the expression of EPHB3 in the goblet-like cells is responsible for their crypt localization, and that the up-regulation of SOX9, a protein that is necessary for Paneth differentiation, represents a compensatory response to the loss of Paneth cells.

Our data support the hypothesis that GATA6 modulates secretory cell differentiation in the ileum by regulating downstream Notch effectors. Specifically, *Ngn3* was down-regulated (Figure 4A), and since NGN3 is required for enteroendocrine differentiation[26] this down-regulation is likely responsible for the observed decrease in enteroendocrine cells. *Dll1* was also significantly down-regulated (Figure 4A), consistent with the expansion of goblet cells when Delta-Notch signaling, and subsequent lateral inhibition of secretory cell differentiation, is disrupted.[23] Conditional deletion of *Gata6* also resulted in an increase in *Spdef* expression (Figure 4). Overexpression of *Spdef* causes an expansion of goblet cells with concomitant decreases in Paneth and enteroendocrine cells,[21] whereas conditional *Spdef* deletion up-regulates *Dll1* and impairs goblet and Paneth cell maturation.[22] Our data are consistent with the hypothesis that GATA6 maintains the balance among secretory cells by inhibiting *Spdef* expression and stimulating both *Dll1* and *Ngn3* expression in secretory progenitors to promote Paneth and enteroendocrine cell differen-

tiation. G*ata6* mRNA abundance was unaffected by either *Gfi1* or *Spdef* deletion or *Spdef* overexpression. Together, these data suggest that GATA6 acts independently of GFI1 and upstream of SPDEF, DLL1 and NGN3 to modulate secretory cell differentiation.

In contrast to the ileum, where intestinal *Gata6* deletion resulted in multiple changes in proliferation, differentiation, and gene expression, in jejunum, intestinal *Gata6* deletion caused only a paradoxical increase in the number of Paneth cells, though with atypical structural features (Figure 6). This suggests that GATA6 is necessary for proliferation, differentiation, and gene expression in ileum, but dispensable for these processes in the jejunum. Emergence of this phenotype in jejunum of double *Gata6/Gata4* conditional knockout mice indicates that GATA4 and GATA6 both share these functions. The increase in Paneth cells in the *G6del* jejunum was unexpected. Others have shown that hormonal changes[27, 28] or massive small intestinal resections[29] increase the number of Paneth cells in the (remaining) small intestine. Thus, it is tempting to speculate that the increase in Paneth cells in the *G6del* jejunum represents a compensatory response to the loss of Paneth cells in the ileum.

Previously, we showed that conditional deletion of *Gata4* results in a jejunum-to-ileum transformation in absorptive enterocyte gene expression.[3, 5, 30] These changes occur in the presence of GATA6, indicating that GATA6 cannot replace GATA4 to regulate specific GATA4 gene targets. Here, we show that in the ileum, GATA6 activates and represses specific absorptive enterocyte genes that are different from the GATA4 targets (Figure 5 and Supplementary Figure 6B). Many of the genes down-regulated in *G6del* mice encode lipid transporters and apolipoproteins. indicating that *Gata6* regulates ileal lipid metabolism. Many of the genes up-regulated by conditional *Gata6* deletion, including *Slc2a1* and *Car1*, are normally more highly expressed in colon than small intestine.[31, 32] Together, these data demonstrate that GATA factors maintain the proximal-distal transcriptome in the small intestine, and that the expression of specific absorptive enterocyte genes is regulated by mechanisms that involve differential recruitment of specific GATA factors.

Members of the GATA family have been shown to function in both specific and overlapping patterns in cardiac myocyte differentiation[33-35] and in lineage commitment in hematopoiesis.[36] Here, we show that in jejunum of *G6del* mice, none of the absorptive enterocyte genes modulated in the ileum of *G6del* mice were altered, indicating that GATA6 is dispensable for their regulation in this region. In the jejunum of the double *Gata6/Gata4* conditional knockout mice, some genes remain unchanged (*Slc2a1* and *Si*, Figure 7E) indicating that both GATA6 and GATA4 are dispensable, while other genes (*Car1* and *Apoa1*, Figure 7E) were altered, indicating that GATA4 can replace GATA6 for their regulation. These data indicate that GATA6 and GATA4 have specific and overlapping functions in the small intestine.

GATA proteins regulate proliferation, cellular differentiation, and gene expression in multiple organs.[2] Here, we show that in distal ileum where GATA4 is not expressed, GATA6 regulates proliferation, secretory cell differentiation, and absorptive enterocyte gene expression. In jejunum, where GATA4 and GATA6 are co-expressed in crypt and villus epithelium, the two transcription factors are redundant for the regulation of most of these processes. We previously revealed spe-

cific functions for GATA4 in jejunal absorptive enterocytes, but were unable to discern a crypt function for this transcription factor. We now believe that GATA4 and GATA6 not only regulate terminal differentiation markers within absorptive enterocytes, but also maintain normal proliferation and cellular differentiation by controlling cell cycle and the differentiation programs of secretory progenitors, explaining their function in crypts.

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GRANTS

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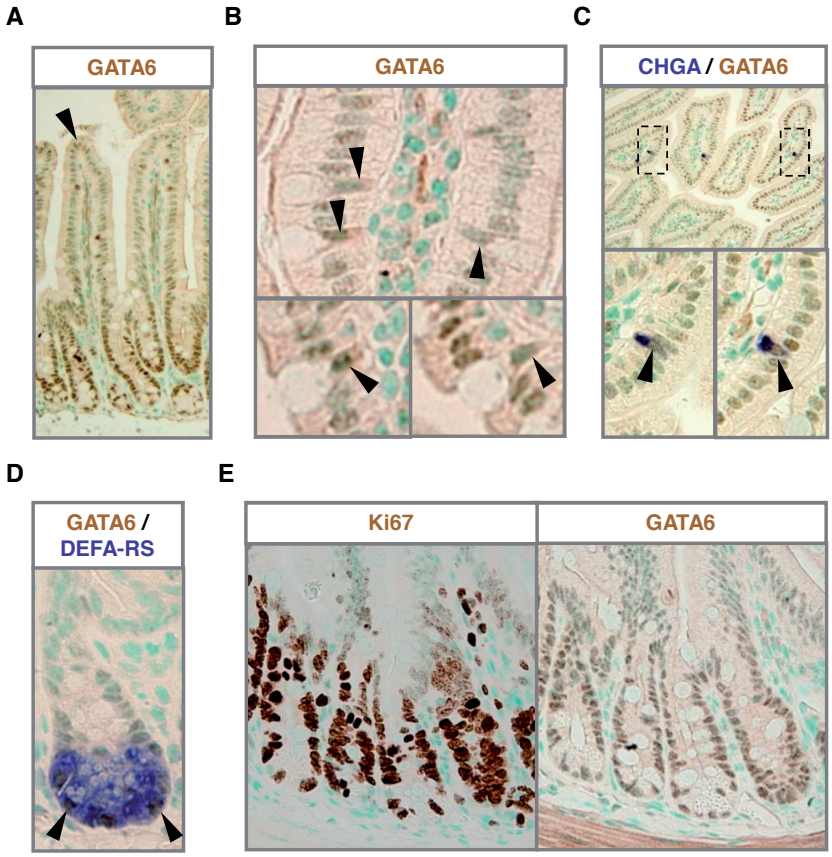
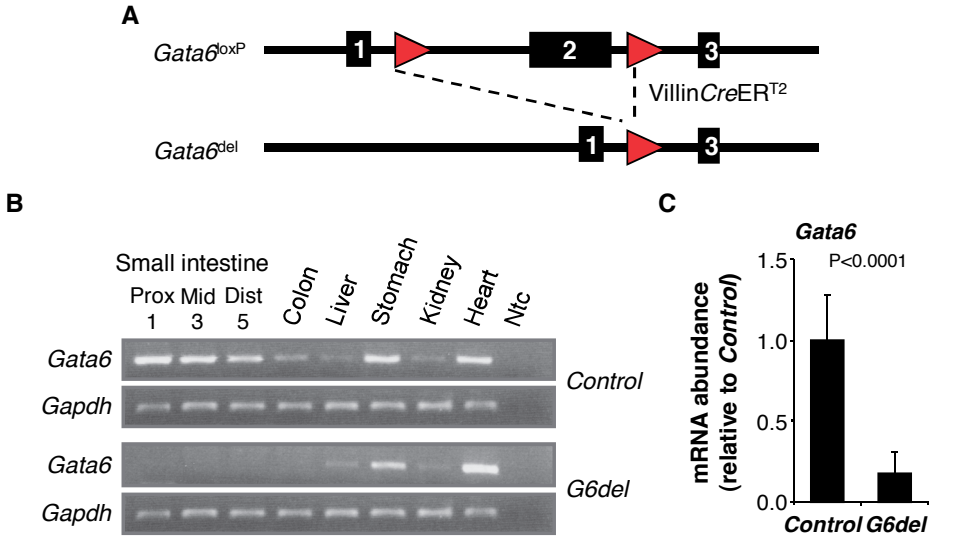
SUPPLEMENTAL FILES

Muc2:	F: 5'-AACTACCACTGTGATGCCAATG-3'
	R: 5'-ACAATGTTGATGCCAGACTCG-3'
Tff3:	F: 5'-AGTGGTCCTGAAGC-3'
	R: 5'-CGATGTGACAGAGG-3'
Lyz:	F: 5'-ACTCCTCCTTGCTTTCTGTC-3'
	R: 5'-GTCGGTGCTTCGGTCTC-3'
Chga:	F: 5'-CACAGCCACCAATACC-3'
	R: 5'-TCTTCCTCCTCCTCTTC-3'
Gcg:	F: 5'-TCTGACGAGATGAGCACCATTCTG-3'
	R: 5'-CTGGCACGAGATGTTGTGAAGATG-3'
Pyy:	F: 5'-CGACAGCGACAGCGAGAAC-3'
	R: 5'-AGGGACAGGGAAATGAACACAC-3'
Defa1:	F: 5'-AGCCAGGAGAAGAGGACCAG-3'
	R: 5'-AGGTTCCATTCATGCGTTCT-3'
Fzd5:	F: 5'-AGTGACCAAGGCAGAG-3'
	R: 5'-GGCATCGGAATAAGACC-3'
Ephb2:	F: 5'-TCATCGCTGTGGTCGTCATTG-3'
	R: 5'-GTCCGCTGGTGTAGTGTTGTAG-3'
c-Myc:	F: 5'-CTCACTGGAACTTACAATCTG-3'
	R: 5'-CAACGCCCAAAGGAAATC-3'
Cd44:	F: 5'-ACCCTCGTTGCCCTTCTC-3'
	R: 5'-TCTGCTGATGTGGATGTGC-3'
Sox9:	F: 5'-GGAGCGACAACTTTAC-3'
	R: 5'-GGCACTTAGCAGAGG-3'
Ngn3:	F: 5'-CTCAGCAAACAGCGAAGAAG-3'
	R: 5'-GGGAAGGTGGGCAGGAC-3'
Gfi1:	F: 5'-ATCGGTGCTGACCCTCGTTT-3'
	R: 5'-AATGTTTGGACCCTCGGATACTCT-3'
Spdef:	F: 5'-ACTGATCTAGGGATACAC-3'
	R: 5'-CGTTTGTGAACAATCCTA-3'
Dll1:	F: 5'-CCTTCAGCAACCCCATCC-3'
	R: 5'-AGCAACCTTCTCCGTAGTAG-3'
Gata6:	F: 5'-CGAGGAATCAAAAGTCAGG-3'
	R: 5'-AGTCAAGGCCATCCACTGTC-3'
Slc2a1:	F: 5'-TATCTCCACACTGTAGTC-3'
	R: 5'-CAGAGTTCGGTATTAGTG-3'
Car1:	F: 5'-GACAGTAGCAACCAATC-3'
	R: 5'-TTCATCAAAACGCCAAG-3'
Apoa1:	F: 5'-GGACTTCTGGGATAACCT-3'
	R: 5'-GCACCTTCTGTTTCACTT-3'
Si:	F: 5'-CAGACCCGTAATCGTTTCC-3'
	R: 5'-AGACCTTGACATCATACAGTG-3'
Gata4:	F: 5'-TTTGAGCGAGTTGGG-3'
	R: 5'-GAATGCGGGTGTGC-3'
Lph:	F: 5'-CAGCGATGCCCACAGGAAAG-3'
	R: 5'-ACGGAGCCCTTGACGAGAG-3'
Asbt:	F: 5'-TTGCCTCTTCGTCTACACC-3'
	R: 5'-CCAAAGGAAACAGGAATAACAAG-3'
Gapdh:	F: 5'-GCCTTCCGTGTTCCTACCC-3'
	R: 5'-TGCCTGCTTCACCACCTTC-3'

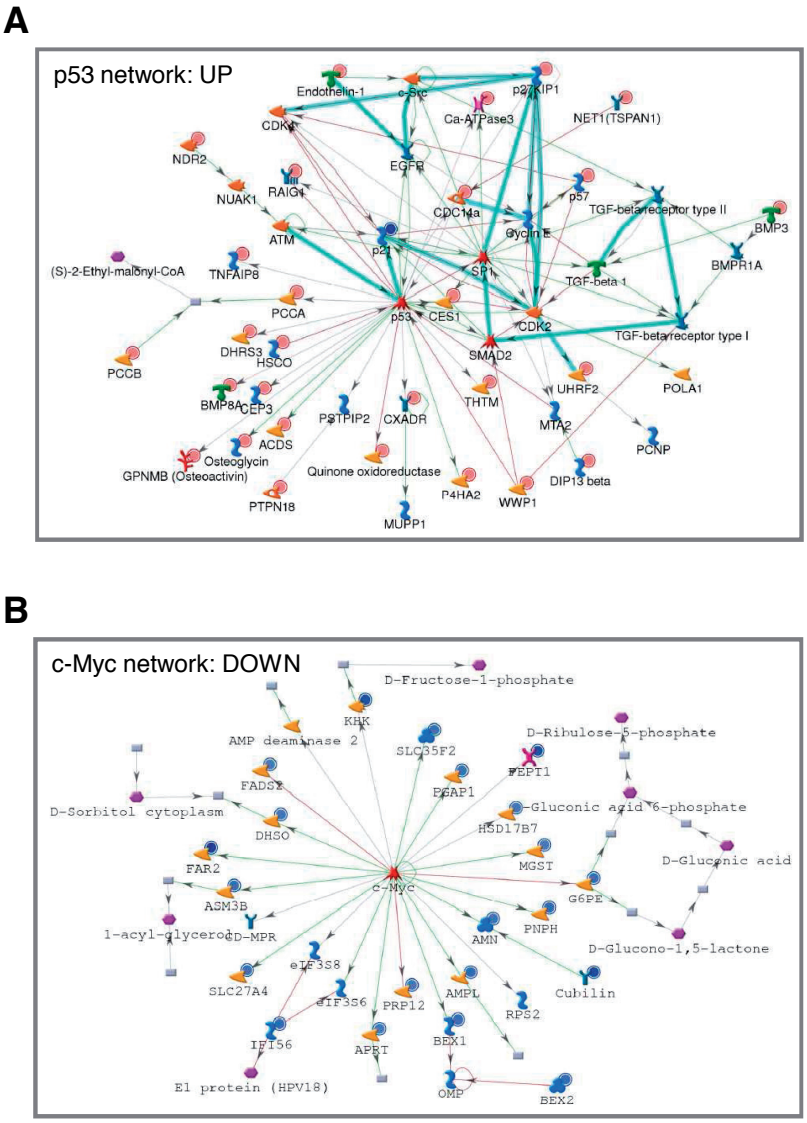
Supplementary Figure 1. Primer sequences used for quantitative reverse transcriptase polymerase chain reactions (qRT-PCR).



Supplementary Figure 2. GATA6 is expressed in all differentiated and proliferating cells in the mature mouse small intestinal epithelium. Immunostaining with rabbit anti-GATA6 (1:50, Santa Cruz) (A-E) revealed that GATA6 is expressed in absorptive enterocytes (A), goblet cells (B), chromogranin A (CHGA)-positive enteroendocrine cells (C), and alpha-defensin related sequence (DEFA-CR)-positive Paneth cells (D) and in Ki67-positive nuclei of proliferating crypt cells as determined on serial sections (E) with the highest staining intensity in the proliferative crypt compartment. Biotinylated secondary antibodies were linked to avidin-horseradish peroxidase or avidin-alkaline phosphatase conjugates (Vector Labs), and visualized using 3,3'-diamino benzidine (DAB) for 2-5 min or 4-nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3indolyl-phosphate (BCIP) for 20-90 min, respectively. Arrowheads indicate GATA6-positive nuclei.

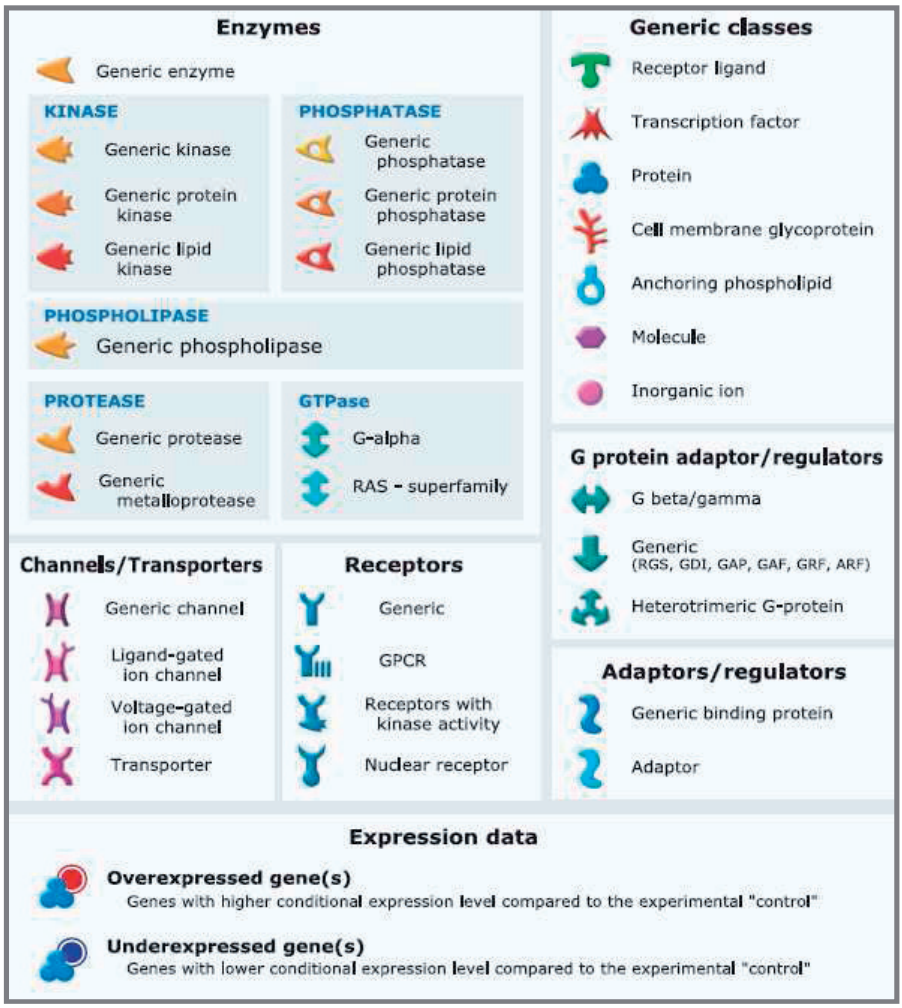


Supplementary Figure 3. Conditional, inducible deletion of *Gata6* in adult mouse intestine. (A) Schematic representation of the *Gata6*^{loxP} and *Gata6*^{del} alleles (Sodhi CP, Li J, Duncan SA. Generation of mice harbouring a conditional loss-of-function allele of *Gata6*. BMC Dev Biol 2006;6:19) used in this study. Villin*Cre*ER^{T2}-mediated recombination of the inserted loxP sites results in the deletion of exon2 of the *Gata6* allele. Boxes with numbers indicate exons. Red arrowheads indicate loxP sites. (B) Semi-quantitative RT-PCR revealed that *Gata6* mRNA is expressed normally in *Control* mice (*Gata6*$^{loxP/loxP}$, Villin*Cre*ER^{T2}–negative) (B, upper panel) and is specifically deleted in the intestine of *G6del* mice (*Gata6*$^{loxP/loxP}$, Villin*Cre*ER^{T2}–positive) (B, lower panel). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA was used as positive control. (C) Quantitative RT-PCR showed a >90% decrease in *Gata6* mRNA abundance in *G6del* as compared to *Control* ileum (n=5 in each group). Primer sequences used: F: 5'-CGAGGAATCAAAAGTCAGG-3', R: 5'-AGTCAAGGCCATCCACTGTC-3'.



Supplementary Figure 4. Intestinal *Gata6* deletion alters gene networks controlling cell proliferation in the mature ileum. Network analyses on microarray data of *Control* and *G6del* ileum (n=3 in each group) revealed transcript level changes after *Gata6* deletion. Significantly differentially expressed transcripts were determined at the 5% FDR level using Significance Analysis of Microarrays (SAM) and interaction networks were developed from the differentially expressed transcripts using Metacore. (A) A network demonstrating an increase in targets of the tumor surpressor gene p53. (B) A network demonstrating a decrease in targets of the proto-oncogene c-MYC. Red circles = up-regulated transcripts; Blue circles = down-regulated transcripts. (C) Legend defining symbols used in the networks. Arrows indicate the direction of the interaction. The definition and accession numbers of the mRNA abbreviations can be found in the microarray data file. (see next page)

C



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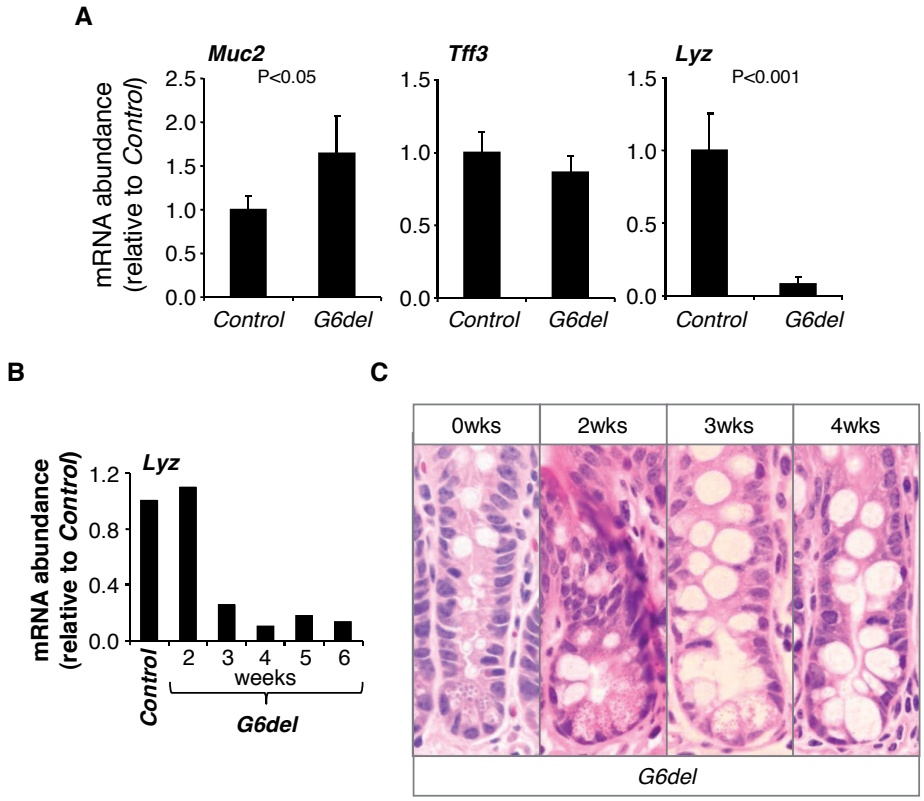
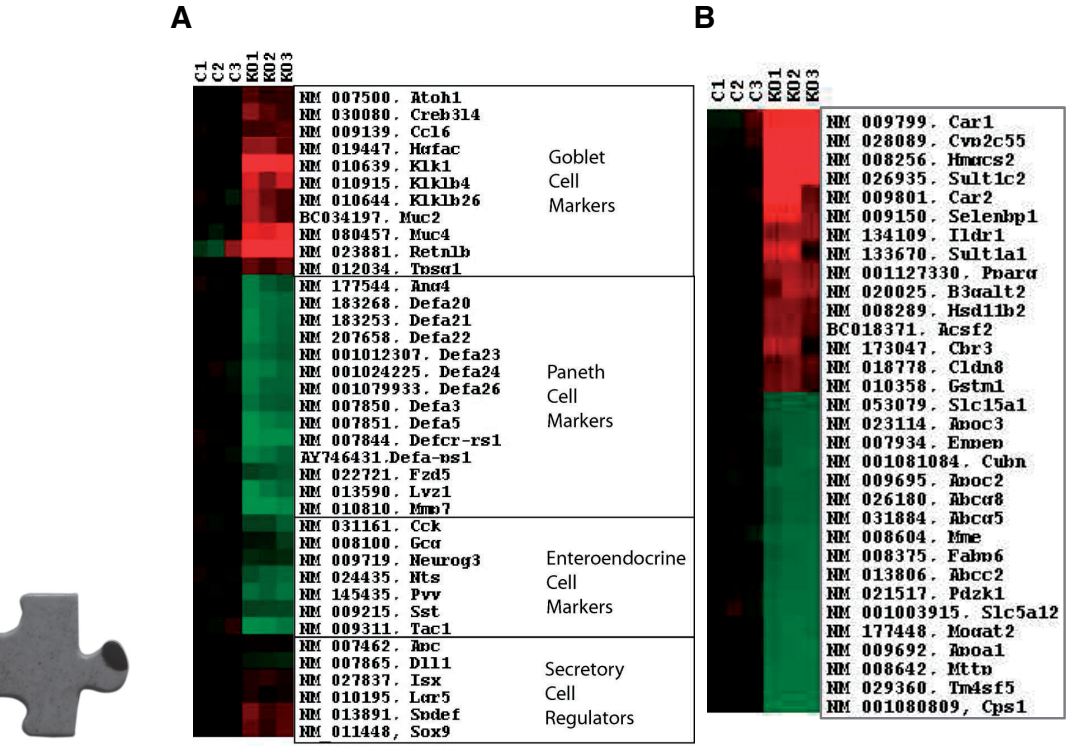
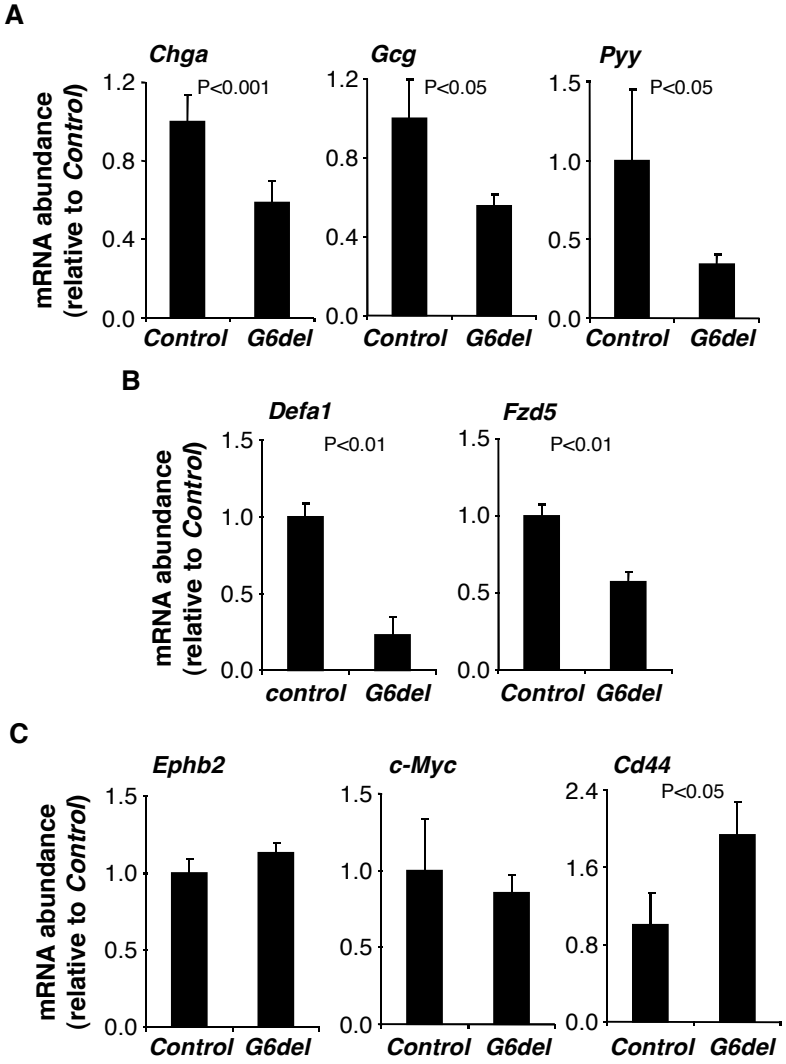


Supplementary Figure 5. Intestinal *Gata6* deletion results in a gradual disappearance of Paneth cells and accumulation of goblet-like cells. (A) Quantitative RT-PCR revealed an increase in mucin 2 (*Muc2),* no change in trefoil factor 3 (*Tff3*) and a decrease in Lysozyme (*Lyz*) mRNA abundance in *G6del* as compared to *Control* ileum (n=5 in each group). (B) Quantitative RT-PCR showed a gradual decrease in *Lyz* mRNA abundance beginning after two weeks, and reaching the lowest level at four weeks after the 5-day tamoxifen treatment (n=1 in each group). (C) Structural analysis by hematoxylin and eosin (H&E) staining on sections harvested 0, 2, 3 and 4 weeks after the 5-day tamoxifen treatment revealed that Paneth cells disappear, while goblet-like cells accumulate gradually over 4 weeks after tamoxifen treatment in the crypts of *G6del* ileum.



Supplemental Figure 6. **Intestinal *Gata6* deletion results in aberrant cellular differentiation.** Microarray analyses on *Control* and *G6del* ileum (n=3 in each group) revealed transcript-level changes after *Gata6* deletion. Significantly differentially expressed transcripts were determined at the 5% FDR level using Significance Analysis of Microarrays (SAM) and then visualized using Gene Cluster and Treeview. (A) Goblet cell markers (increased), Paneth and endocrine cell markers (decreased), and secretory lineage regulators (mixed response). (B) Markers for enterocytes (up and down regulated). Green = down-regulated; Red = up-regulated; level of color saturation = magnitude of regulation compared to *Control* ileum levels.



Supplemental Figure 7. **Intestinal *Gata6* deletion results in reductions in enteroendocrine cell markers and Paneth cell-specific Wnt signaling.** Quantitative RT-PCR revealed (A) decreases in the mRNA abundances of the enteroendocrine cell markers chromogranin A (*Chga*), glucagon (*Gcg*) and peptide YY (*Pyy*), (B) decreases in the mRNA abundance of the Wnt target alpha-defensin 1 (*Defa1*) and the Paneth cell-specific Wnt receptor frizzled 5 (*Fzd5*), and (C) no change in the mRNA abundance of the Wnt targets eph receptor B2 (*Ephb2*) and myelocytomatosis oncogene (*c-Myc*) and an increase in the mRNA abundance of the Wnt target CD44 antigen (*Cd44*) in *G6del* as compared to *Control* ileum (n=5 in each group).

Mice

Previously established and confirmed *Gata6*$^{loxP/loxP}$, *Gata4*$^{flap/flap}$ and transgenic Villin*Cre*ER^{T2} mice[3, 5-7] were used in this study to produce conditional, inducible deletion of *Gata6* or both *Gata6* and *Gata4* in the intestinal epithelium. DNA was obtained from tail biopsies and genotypes were determined as described.[3, 5, 7, 8] Test animals included *Gata6*$^{loxP/loxP}$, Villin*Cre*ER^{T2}-positive (*G6del*) and *Gata6*$^{loxP/loxP}$, *Gata4*$^{flap/flap}$, Villin*Cre*ER^{T2}-positive (*G6G4del*) mice. *Gata6*$^{loxP/loxP}$, Villin*Cre*ER^{T2}-negative and *Gata6*$^{Wt/Wt}$, Villin*Cre*ER^{T2}-positive mice were indistinguishable and were used interchangeably (*Control*). Mice 6-8 wk of age were treated with a single injection of tamoxifen (100 mg/ml, Sigma-Aldrich, St. Louis, MO) for 5 consecutive days as described.[3, 5, 8] Tissue was collected 28 days after the last injection, unless otherwise indicated. Approval was obtained from the Institutional Animal Care and Use Committee.

Tissue isolation

Mice were dissected as previously described[3] and intestinal segments were prepared for sectioning and RNA isolation. Samples of ileum were taken from distal small intestine adjacent to the ileocecal valve whereas samples of jejunum were taken from the geometric center of the small intestine. In selected mice, bromodeoxyuridine (BrdU) (0.1 ml of 10 mg/ml) was injected one hour prior to dissection.

Sectioning, staining, immunohistochemistry, and electron microscopy

Intestinal segments were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 4 h, dehydrated, embedded in paraffin and sectioned as previously described.[3] For immunostaining, primary antibodies used were as follows: rabbit anti-Ki67 (1:200, Santa Cruz), mouse anti-BrdU (1:250, Neomarkers), goat anti-eph receptor B3 (EPHB3) (1:50, R&D Systems), rabbit anti-mucin 2 (MUC2) (1:200, Santa Cruz), rabbit anti-trefoil factor 3 (TFF3) (1:2000, gift from Dr. D. Podolsky, University of Texas, Southwestern), rabbit anti-lysozyme (LYZ) (1:100, Zymed) and goat anti-alpha-defensin related sequence (DEFA-RS) (1:1000) (gifts from Dr. A. Ouellette, University of Southern California), rabbit anti-chromogranin A (CHGA) (1:2000, Novacastra Laboratories Ltd.), goat anti-carbonic anhydrase 1 (CAR1) (1:200, Santa Cruz), goat anti-sucrase isomaltase (SI) (1:200, Santa Cruz), and rabbit anti-SAM pointed domain containing ets transcription factor (SPDEF) (1:5000, a gift from Dr. J. Whitsett, Cincinnati Children's Hospital Medical Center). Secondary antibodies included: donkey anti-goat IgG, donkey anti-rabbit IgG and donkey anti-mouse IgG (all from Vector Labs), and Alexa Flour 488 anti-rabbit IgG and Alexa Flour 594 anti-goat IgG (Invitrogen). Sections were counterstained with alcian blue, methyl green, and/or hematoxylin.

For electron microscopy (EM), intestinal segments were cut longitudinally along the mesentery and pinned onto a paraffin dish, washed with ice-cold PBS and fixed in 1.25% gluteraldehyde, 4% formaldehyde, 0.1M cacodylic buffer, pH 7.4 (cacodylic acid sodium salt trihydrate, Ted Pella

Inc, Redding, CA, USA) 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

From carefully oriented sections, the crypt-villus junction was determined, and 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. From sections stained with alcian blue, the total number of positive cells on villi was determined as a percentage of total epithelial cells. From sections stained with alcian blue or for Ki67, the total number of positive cells in crypts was determined as total number per crypt. From sections stained for CHGA, the average number of positive cells expressed as a fraction of total epithelial cells (villi and crypts) from a minimum of 5000 epithelial cells was determined. For all determinations, a minimum of six villi or six crypts per slide were analyzed. All determinations were blinded and conducted on a minimum of 5 test and 5 control mice.

RNA isolation and gene expression analysis

RNA was isolated from 0.5 to 1.0 cm intestinal segments as described previously.[3, 5, 8] Gene expression was assessed in two ways. First, specific messenger RNA (mRNA) abundances were determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)[3, 5, 8] using validated primer pairs (Supplementary Figure 1). Glyceraldehyde-3-phosphate dehydrogenase mRNA abundance was used to normalize the data. Second, ileal mRNA was 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 and standard Affymetrix protocols for labeling and hybridization (Affymetrix Inc., Santa Clara, CA). Quality control assessments of chips were conducted using the Affymetrix Gene expression console and chips were normalized using the Robust Multi-array Analysis procedure.[37] Only the 19,434 probe sets with full-length transcript support were used for analysis. Differences between *Control* and *G6del* samples was determined using Significance Analysis for Microarrays and differential expression was determined at the 5% false detection rate level.[38] A list of differentially expressed transcripts chosen to reflect specific cell populations was examined by hierarchical clustering using Gene Cluster and TreeView.[39] Functional analysis of the differential expression was conducted using Metacore (GeneGo Inc., St. Joseph, MI); this included pathway analysis, gene ontology term enrichment, and network analysis.

Statistical analyses

Data are expressed as mean ± SD. Statistically significant differences were determined by the two-tailed Student's *t* test or analysis of variance followed by the Tukey-Kramer multiple comparison test. Differences were considered statistically significant at $P<0.05$.

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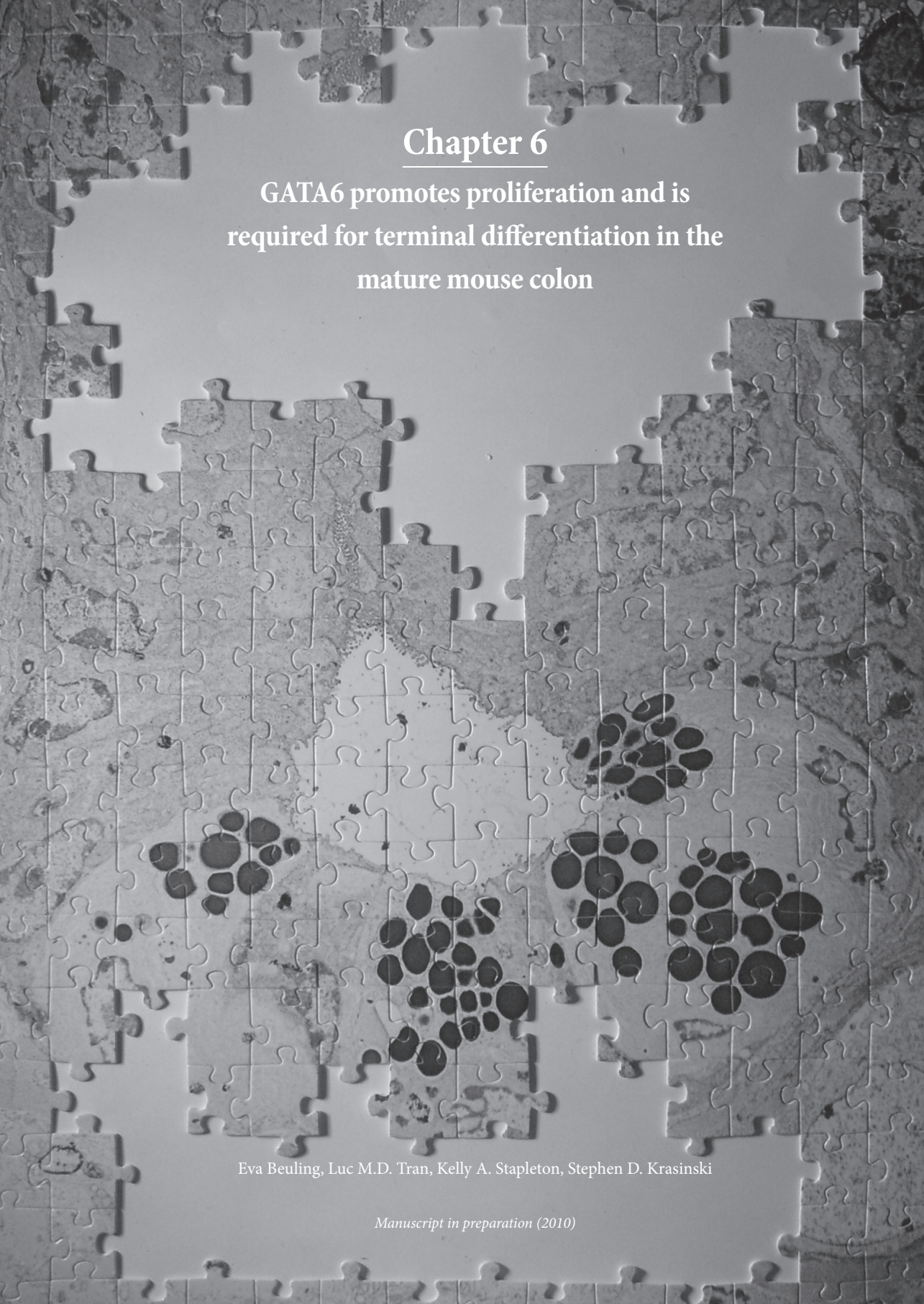

Chapter 6

GATA6 promotes proliferation and is required for terminal differentiation in the mature mouse colon

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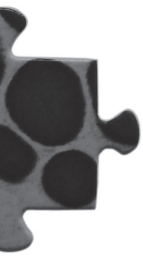
ABSTRACT

GATA6 is a member of the GATA family of transcription factors that play key roles in proliferation, differentiation and gene regulation in multiple organs. GATA6 is expressed throughout the crypt and villus epithelium of the small intestine, where it regulates proliferation, secretory lineage differentiation, and absorptive enterocyte gene expression. GATA6 is also expressed in colon and regulates colon-specific genes in vitro, but its function in this organ in vivo is currently unknown. We hypothesize that in parallel to its functions in the small intestine, GATA6 regulates proliferation, differentiation and colonocyte gene expression in colon. To test this hypothesis, *Gata6* was specifically deleted throughout the small and large intestine of adult mice. In the colon, *Gata6* deletion resulted in a reduction in the number of Ki67-positive cells and BrdU staining, and a reduction in the mRNA abundance and immunostaining intensity for the goblet cell marker mucin 2 in the lower half of the colonic crypts. Furthermore, the mRNA abundances of the enteroendocrine markers secretin, cholecystokinin and glucagon, and in the number of glucagon-like peptide 1-producing enteroendocrine cells were reduced, whereas the mRNA abundance of peptide YY and the number of peptide YY-producing enteroendocrine cells were increased in the colonic crypts after *Gata6* deletion. In the absence of GATA6, the messenger RNA abundances of the colonocyte markers carbonic anhydrase 1 and solute carrier family 9, member 2 and 3, reduced while other colonocyte genes remained unchanged. These data demonstrate that GATA6 is necessary for the maintenance of proliferation, the terminal differentiation of goblet and enteroendocrine cells, and colonocyte gene expression in the mature mouse colon.



INTRODUCTION

The mammalian colon is lined by a highly differentiated epithelium comprised of specialized absorptive and secretory cells with cell distribution and gene expression patterns designed to facilitate the absorption of large quantities of water. The colonic epithelium is maintained through a process of continuous cellular renewal. Stem cells located at the base of the crypts[1] produce 14-21 transient amplifying cells per hour[2] that give rise to the differentiated cell types of the colon, including absorptive colonocytes, goblet cells and enteroendocrine cells. The colonic epithelium turns over in four to six days.[2] Controlled renewal of the epithelium with precise cell distribution and gene expression patterns is essential for colonic function, and is controlled by molecular mechanisms that are only beginning to be elucidated.

GATA factors are evolutionarily conserved zinc finger transcription factors that play key roles in proliferation, differentiation and gene regulation in multiple organs.[3] GATA4 and GATA6 are expressed in the mature gastrointestinal tract in partially overlapping patterns. GATA4 is expressed in the proximal 85% of the small intestine, but is absent from the distal ileum and colon. Conditional knockout studies have shown that GATA4 defines key functional differences between jejunum and ileum by activating jejunum-specific genes and repressing ileum-specific genes in absorptive enterocytes of jejunum.[4-6] GATA6 is co-expressed with GATA4 in the proximal small intestine and is also expressed in the ileum and colon. In the ileum, GATA6 plays critical roles in proliferation, Paneth cell differentiation, enteroendocrine cell commitment and absorptive enterocyte gene expression (Chapter 5). In jejunum, GATA4 is redundant for most, but not all of the functions of GATA6 (Chapter 5).

GATA6 is expressed in the mature mouse[7, 8] and human[9] colonic epithelium, and activates in vitro the promoters of specific colonic genes,[10, 11] but the exact function of GATA6 in the mature colon in vivo is currently unknown. In the present study, we utilized a previously established inducible, intestine-specific *Gata6* deletion model (Chapter 5) to define the function of GATA6 in the colon. We found that conditional deletion of *Gata6* results in alterations in proliferation, secretory cell differentiation, and colonocyte gene expression paralleling GATA6 regulation in the small intestine. However, in the colon GATA6 regulates some of these processes differently than in the small intestine.

MATERIALS AND METHODS

Mice

Previously established and confirmed *Gata6*$^{loxP/loxP}$, Villin*Cre*ER^{T2}-positive mice were used in this study to produce conditional, inducible deletion of *Gata6* in the intestinal epithelium (Chapter 5). DNA was obtained from tail biopsies and genotypes were determined by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using previously validated primers.[5, 12, 13]

Test *Gata6*$^{loxP/loxP}$, Villin*Cre*ER^{T2}-positive (*G6del*) and control *Gata6*$^{loxP/loxP}$, Villin*Cre*ER^{T2}-negative and *Gata6*$^{wt/wt}$, Villin*Cre*ER^{T2}-positive mice 6-8 wk of age were treated with tamoxifen as described (Chapter 5), and tissue was collected 28 days after the last injection. Control *Gata6*$^{loxP/loxP}$, Villin-*Cre*ER^{T2}-negative and control *Gata6*$^{wt/wt}$, Villin*Cre*ER^{T2}-positive mice were indistinguishable and were used interchangeably (*Control*). Approval was obtained from the Institutional Animal Care and Use Committee.

Tissue isolation

Mice were dissected as previously described[5, 12] and colonic segments 2 cm in length were taken ~3 cm distal to the cecum. The segments were sagittally cut in half, and 1 cm was prepared for sectioning and 1 cm for RNA isolation.

Sectioning, Immunohistochemistry and Immunofluorescence

Colonic segments were fixed in 4% paraformaldehyde in PBS for 4 h, dehydrated, embedded in paraffin and sectioned as previously described.[5] For immunostaining, primary antibodies used were as follows: rabbit anti-GATA6 (1:50, Santa Cruz), goat anti-glucagon-like peptide 1 (GLP1) (1:100), goat anti-peptide YY (PYY) (1:50), rabbit anti-Ki67 (1:200, Santa Cruz), mouse anti-bromodeoxyuridine (BrdU) (1:250, Neomarkers), mouse anti-β-catenin (1:200, BD Biosciences), rabbit anti-mucin 2 (MUC2) (1:200, Santa Cruz), rabbit anti-trefoil factor 3 (TFF3) (1:2000, gift from Dr. D. Podolsky, University of Texas, Southwestern), rabbit anti-chromogranin A (CHGA) (1:2000, Novacastra Laboratories Ltd Secondary antibodies included: for immunohistochemistry biotinylated donkey anti-rabbit IgG, donkey anti-goat IgG and donkey anti-mouse IgG (all from Vector Labs), and for immunofluorescence, Alexa Flour 488 anti-rabbit IgG and Alexa Flour 594 anti-goat IgG (Invitrogen). For immunohistochemistry, biotinylated secondary antibodies were linked to avidin-horseradish peroxidase or avidin-alkaline phosphatase conjugates (Vector Labs), and visualized using 3,3'-diamino benzidine (DAB) for 2-5 min or 4-nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3indolyl-phosphate (BCIP) for 20-90 min, respectively. For selected sections, the tissue was lightly counterstained with methyl green.

RNA isolation and gene expression analysis

RNA was isolated from 1 cm of colonic segments as described previously.[5, 12] Messenger RNA (mRNA) abundances were determined by real-time RT-PCR[5, 12] using validated primer pairs (sequences available upon request). Glyceraldehyde-3-phosphate dehydrogenase mRNA abundance was measured for each sample and used to normalize the data. All data were expressed relative to the mean value of *Control* colon.

Enteroendocrine cell counting

Co-immunofluorescence was performed as described above for CHGA and GLP1 or CHGA and PYY. In multiple microscopic fields the number of CHGA-positive cells was determined (green

filter). In the same field, the number of GLP1-positive or PYY-positive cells (red filter) was then determined. A minimum of 100 CHGA-positive cells were counted per animal, and 4 animals in each group were included.

Statistical analyses

Data are expressed as mean ± SD. Statistically significant differences were determined by the two-tailed Student's *t* test. Differences were considered statistically significant at $P<0.05$.

RESULTS

Inducible Villin*Cre*ER^{T2}-mediated recombination in *Gata6*$^{loxP/loxP}$ mice results in *Gata6* deletion in the colon

GATA6 was readily detected in the nuclei of colonocytes and goblet cells (Figure 1A-C), as previously shown in humans.[9] GATA6 was also present in glucagon-like peptide 1 (GLP1)- (Figure 1D-G) and peptide YY (PYY)-positive (Figure 1H-K) enteroendocrine cells. GATA6 was also detected in the nuclei of Ki67-positive cells in the proliferative compartment as determined by serial sections (Figure 1L, M). Together these data show that GATA6 is expressed in all differentiated and proliferating cells in the mature mouse colonic epithelium.

We have previously shown by semi-quantitative RT-PCR that Villin*Cre*ER^{T2}-mediated recombination in mice homozygous for the *Gata6*^{loxP} allele[13] results in deletion of *Gata6* specifically in small and large intestine (Chapter 5). *Gata6* mRNA abundance was reduced >90% in *Gata6*$^{loxP/loxP}$, Villin*Cre*ER^{T2}-positive (*G6del*) colon as compared to *Gata6*$^{loxP/loxP}$, Villin*Cre*ER^{T2}-negative or *Gata6*$^{wt/wt}$, Villin*Cre*ER^{T2}-positive (*Control*) colon, as determined by real-time RT-PCR ($P<0.0001$) (Figure 2A). Further, GATA6 immunostaining intensity was reduced in the *G6del* colon as compared to *Control* colon (Figure 2B, C). Together, these data demonstrate that GATA6 is efficiently deleted in mouse colon after tamoxifen treatment verifying the inducible, intestine-specific *Gata6* deletion model.

GATA6 promotes proliferation in the mature mouse colon

To determine the effect of intestinal *Gata6* deletion on proliferation in the colonic epithelium, we counted the number of Ki67-positive cells (Figure 3A, B) per total epithelial cells and found a 39% reduction in *G6del* colon as compared to *Control* colon ($P<0.01$) (Figure 3C). Immunostaining for BrdU on tissue samples harvested 1h after BrdU injection demonstrated fewer cells containing BrdU label in *G6del* colon as compared to *Control* colon (Figure 3D, E), confirming a reduction in proliferating cells. Since the Wnt signaling pathway is a key pathway that promotes proliferation in the mature mouse colon,[14] we hypothesized that the decrease in proliferation in the *Gata6* deficient colon (See Figure 3) is due to a decrease in Wnt signaling, and that nuclear β-catenin, the hallmark of activated Wnt signaling within cells,[15] would be decreased in the *G6del*

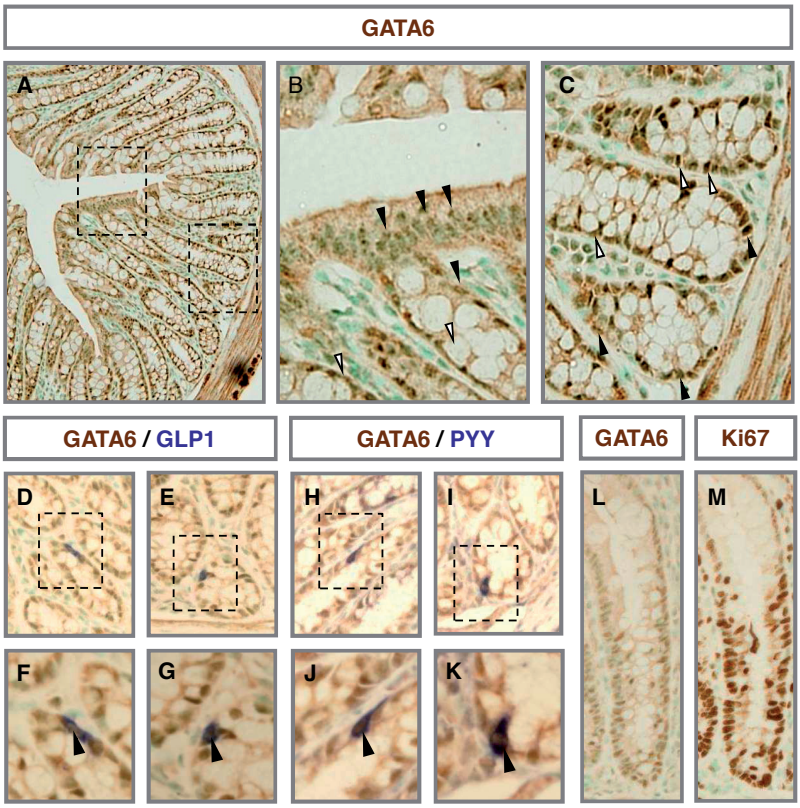


Figure 1. GATA6 is expressed in all differentiated and proliferating cells in the mature mouse colonic epithelium. (A-M) Immunostaining reveals that GATA6 is expressed in colonocytes and goblet cells (A-C), glucogon-like peptide 1 (GLP1)- (D-G) and peptide YY (PYY)-positive (H-K) enteroendocrine cells and in Ki67-positive nuclei of proliferating crypt cells as determined on serial sections (L, M). Arrowheads indicate GATA6-positive nuclei. White arrowheads indicate goblet cells with GATA6-positive nuclei.

colon. However, nuclear β-catenin was increased in the proliferative compartment at the crypt base in *G6del* colon as compared to *Control* colon (Figure 4A, B). Further, the mRNA abundances of the Wnt signaling targets eph receptor B3 (*EphB3*) and myelocytomatosis oncogene (*c-Myc*) were not significantly different between *G6del* and *Control* colon (Figure 4C). These data suggest that GATA6 promotes proliferation in the mature mouse colon independently of Wnt signaling.

GATA6 promotes the differentiation of goblet cells in the mature mouse colon

To determine the effect of intestinal *Gata6* deletion on goblet cell differentiation in the colon, we measured the mRNA abundances of krüppel-like factor 4 (*Klf4)* that is necessary for goblet lineage differentiation in colon,[16] and the goblet cell markers trefoil factor 3 (*Tff3*) and *Muc2*. We failed to find differences in the mRNA abundances of *Klf4* and *Tff3* between *G6del* and *Control*

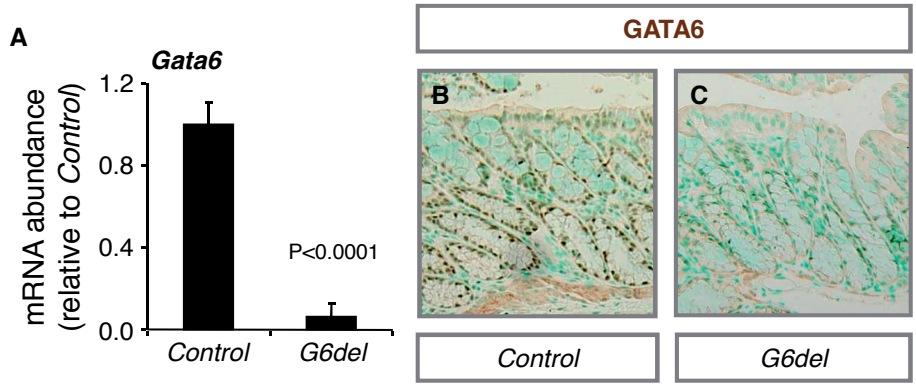


Figure 2. Conditional, inducible deletion of *Gata6* in adult mouse colon. (A) Real-time RT-PCR shows a >90% decrease in *Gata6* mRNA abundance in *G6del* as compared to *Control* colon (n=7 in each group). (B, C) Immunohistochemistry reveals that GATA6 protein is ablated in *G6del* colon.

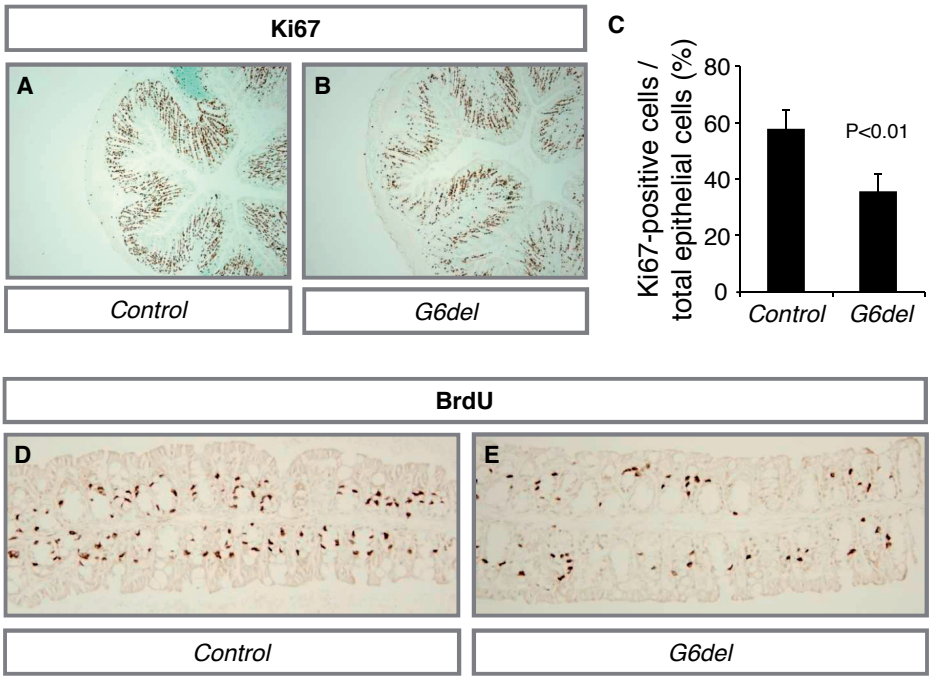


Figure 3. GATA6 promotes proliferation in the mature mouse colon. (A-C) Counts on sections stained with Ki67 (A, B) reveal a decrease in the number of Ki67-positive cells per total number of epithelial cells in *G6del* as compared to *Control* ileum (C) (n=6 per animal, 6 animals in each group, 3 individual investigators). (D, E) Immunohistochemistry reveals a decrease in bromodeoxyuridine (BrdU) staining after intestinal *Gata6* deletion.

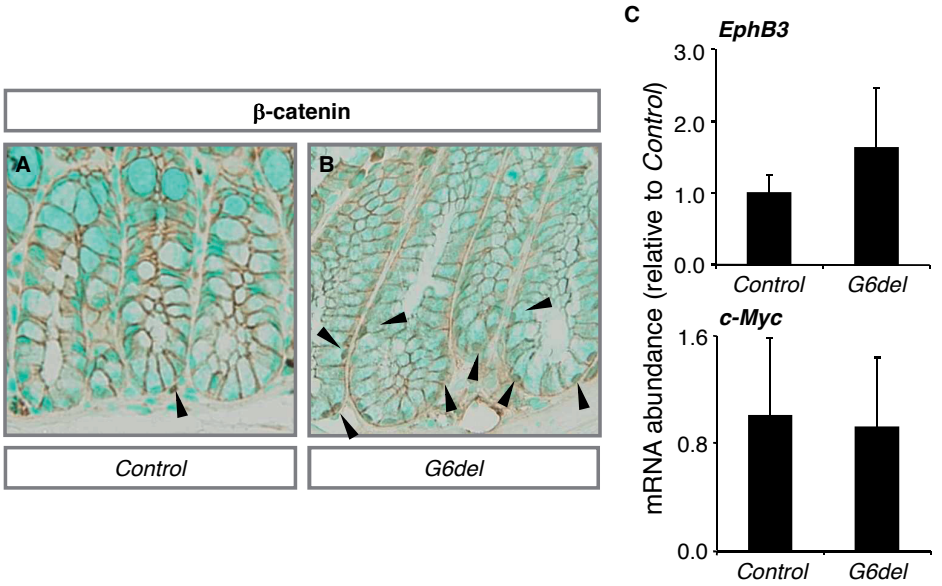


Figure 4. GATA6 promotes proliferation in the mature mouse colon independently of Wnt signaling. (A, B) Immunostaining reveals that nuclear β-catenin is increased in cells in the proliferative compartment after intestinal *Gata6* deletion. (C) Real-time RT-PCR shows no difference in eph receptor B3 (*EphB3*) and myelocytomatosis oncogene *(c-Myc)* mRNA abundances between *G6del* and *Control* colon (n=5 in each group).

colon (Figure 5A), suggesting that goblet cell differentiation remains grossly intact. However, *Muc2* mRNA abundance was reduced 64% (P<0.01) (Figure 5A), suggesting that although goblet cells are committed, they are not fully differentiated. The intensity of MUC2 immunofluorescence was decreased mainly in the lower half of the crypts (Figure 5B, C) and TFF3-positive goblet cells localized higher up the crypts (Figure 5D, E) in *G6del* as compared to *Control* colon consistent with a delayed maturation process and/or an increased migration rate of committed goblet cells. Together, these data suggest that GATA6 activates MUC2 expression, promotes goblet cell differentiation and/or inhibits the migration rate of committed goblet cells in the mature mouse colon.

GATA6 defines the terminal differentiation of enteroendocrine sublineages in the mature mouse colon

To determine the effect of intestinal *Gata6* deletion on the enteroendocrine cells in the colon we measured the mRNA abundances of different hormones produced in the colon. The mRNA abundances for the pan-endocrine cell marker chromogranin A (*Chga*) and the endocrine cell marker tachykinin 1 (*Tac1*) (also known as Substance P) (Figure 6) and the number of CHGA-positive cells (data not shown) were indistinguishable between *G6del* and *Control* colon, suggesting that enteroendocrine cell commitment remains intact after *Gata6* deletion. However, secretin

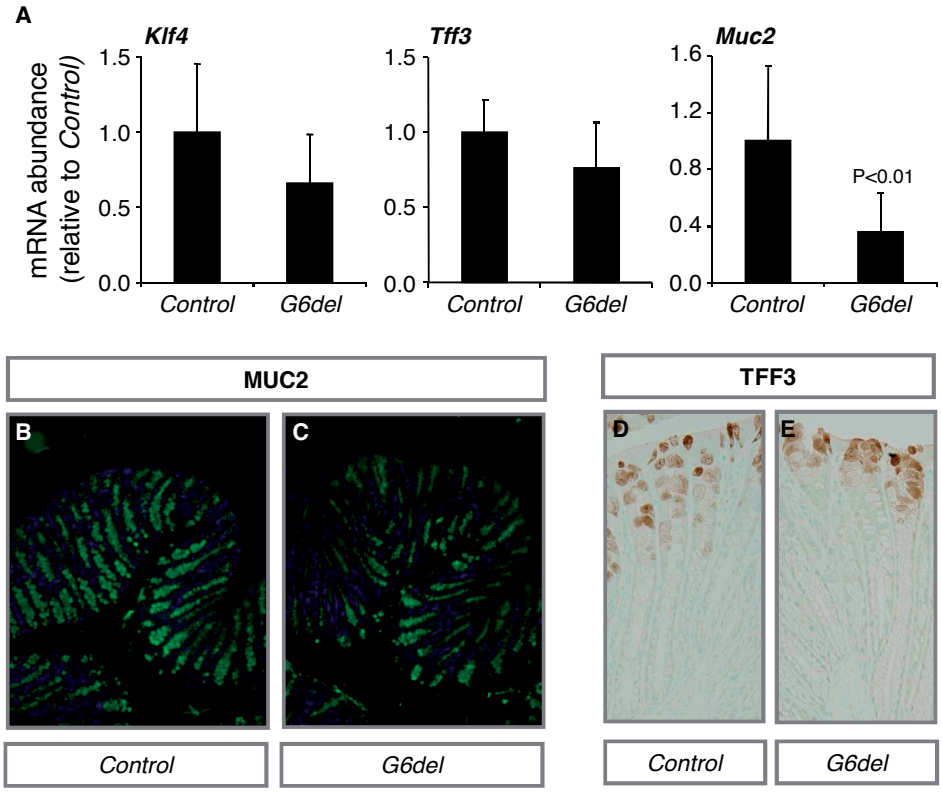


Figure 5. GATA6 promotes the terminal differentiation of goblet cells in the mature mouse colon. (A) Real-time RT-PCR shows a decrease in mucin 2 (*Muc2*), mRNA abundance in *G6del* as compared to *Control* colon (n=7 in each group). (B, C) Immunostaining reveals a reduction in MUC2-positive goblet cells mainly in the lower half of the crypts in *G6del* as compared to *Control* colon. (D, E) Immunostraining reveals that trefoil factor 3 (TFF3)-positive goblet cells localize higher up the crypts in *G6del* as compared to *Control* colon.

(*Sct*), cholecystokinin (*Cck*), and glucagon (*Gcg*) mRNA abundances were reduced 81% (P<0.05), 91% (P<0.0001), and 94% (P<0.0001), respectively, whereas peptide YY (*Pyy*) was increased 79% (P<0.05) in *G6del* colon as compared to *Control* colon (Figure 6).We counted the number of GLP1- and PYY-expressing cells as percentages of the number of CHGA-expressing cells. Co-immunofluorescence with GLP1 and CHGA (Figure 7A-F), and PYY and CHGA (Figure 7H-M) demonstrated a 92% reduction in the number of GLP1-expressing cells per total number of CHGA-expressing cells (P<0.0001) (Figure 7G), and a 72% increase in the number of PYY-expressing cells per total number of CHGA-expressing cells (P<0.01) (Figure 7N) in *G6del* as compared to *Control* colon. These data indicate that GATA6 determines enteroendocrine sublineages by regulating their terminal differentiation.

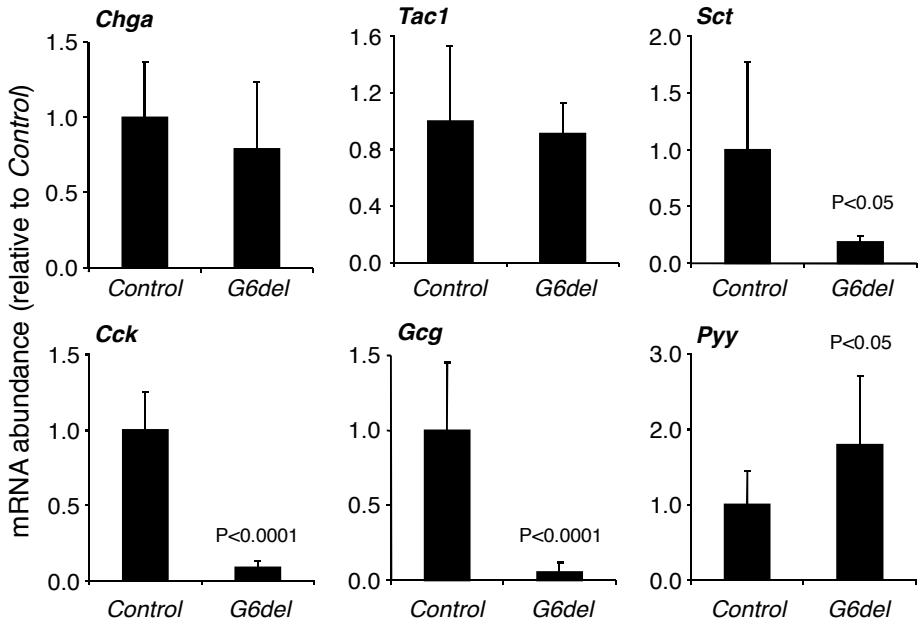


Figure 6. Intestinal *Gata6* deletion results in an enteroendocrine sublineage shift in the mature mouse colon. Real-time RT-PCR shows a decreases in secretin (*Sct*), cholecystokinin (*Cck*) and glucagon (*Gcg*), and an increase in peptide YY (*Pyy*) mRNA abundance in *G6del* as compared to *Control* colon (n=7 in each group).

GATA6 is required for the expression of specific colonocyte genes in the mature mouse colon

To determine the effect of *Gata6* deletion on colonocyte gene expression, we measured the mRNA abundances of terminal differentiation markers for colonocytes. We did not find differences in the mRNA abundance of the colonocyte markers anion exchanger 1 (*Ae1*, also known as *Slc4a1*) and 2 (*Ae2*, also known as *Slc4a2*); ATPase, Na+/K+ transporting, polypeptide beta 1 (*Atp1b1)* and 3 (*Atp1b3*); solute carrier family 2 (facilitated glucose transporter), member 1 (*Slc2a1*); and carbonic anhydrase 2 (*Car2*) between *G6del* and *Control* colon (data not shown and Figure 8), suggesting that colonocyte commitment and the expression of specific colonocyte genes are not affected by conditional *Gata6* deletion. However, we found 89% (P<0.05), 78% (P<0.01) and 79% (P<0.001) reductions in the mRNA abundances of the colonocyte markers carbonic anhydrase 1 (*Car1*), solute carrier family 9 (sodium/hydrogen exchanger), member 2 and 3 (*Slc9a2* and *Slc9a3*, also known as *Nhe2* and *Nhe3*), respectively, in *G6del* colon as compared to *Control* colon (Figure 8), demonstrating that GATA6 activates the expression of specific colonocyte genes in the mature mouse colon. Interestingly, these three genes are up-regulated by *Gata6* deletion in ileum (Chapter 5), indicating that GATA6 regulates these genes differently in small intestine and colon.

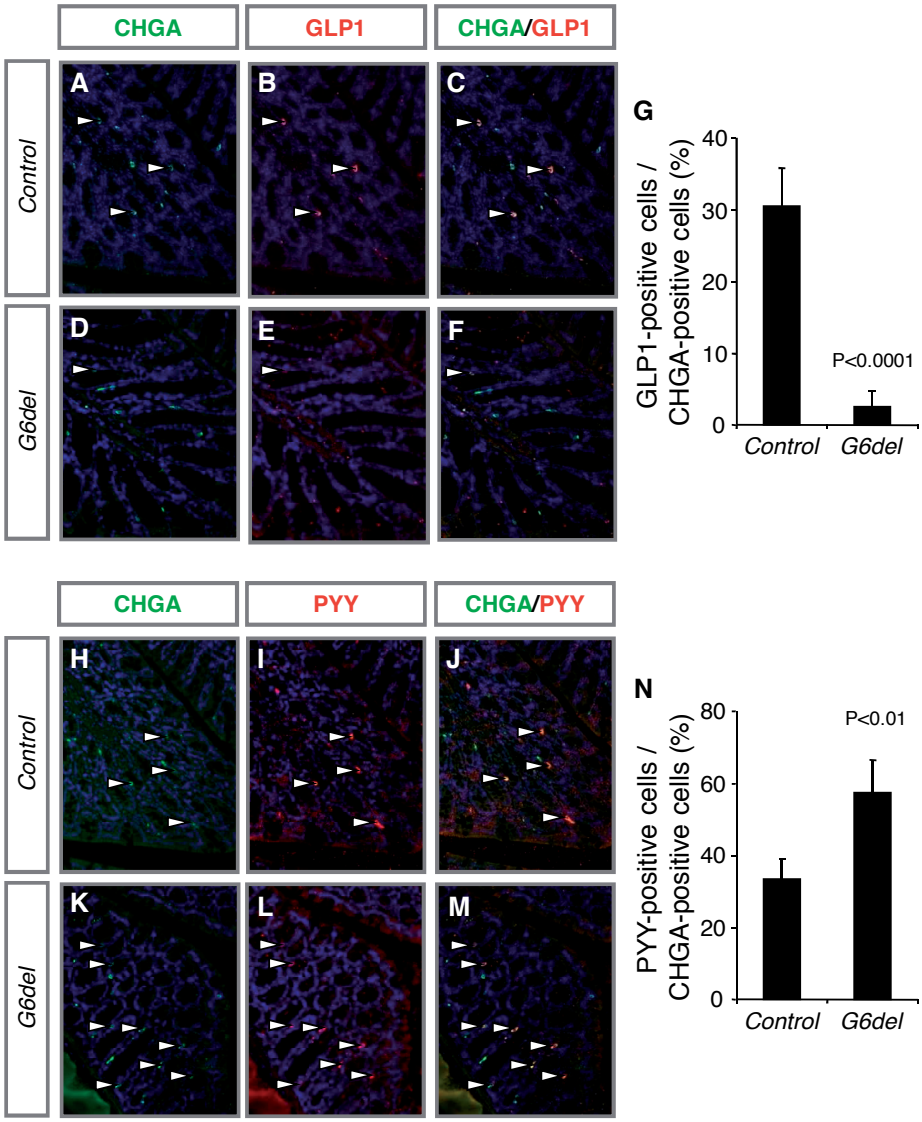


Figure 7. GATA6 promotes glucagon-like peptide 1- and inhibits peptide YY-expressing enteroendocrine cells. (A-N) Counts on sections stained with glucogon-like peptide 1 (GLP1) and chromogranin A (CHGA) (A-F), or peptide YY (PYY) and CHGA (H-M)) reveal a decrease in the number of GLP1-positive cells (G) and an increase the number of PYY-positive cells (N) per total number of CHGA-positive cells in *G6del* as compared to *Control* ileum (n=4 in each group). Arrowheads indicate positive cells.

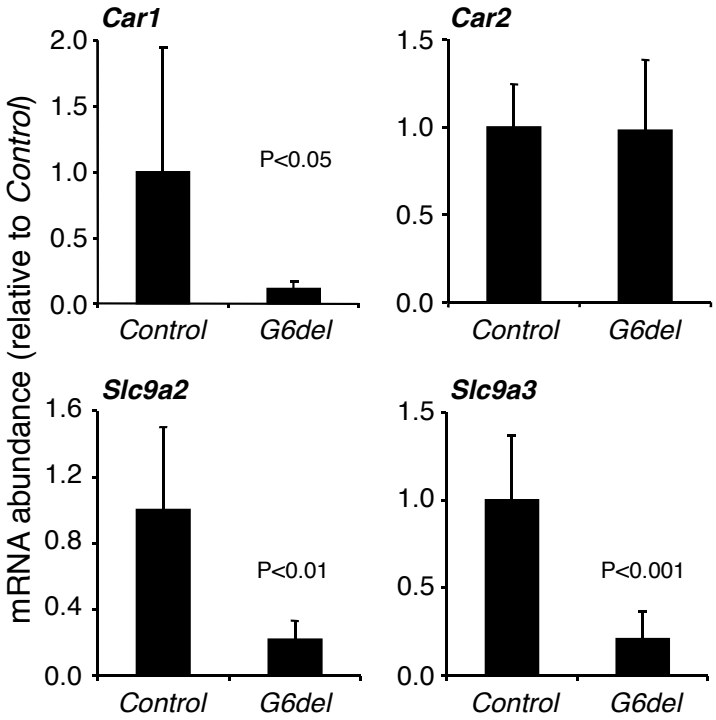


Figure 8. GATA6 is required for the expression of specific colonocyte genes in the mature mouse colon. Real-time RT-PCR shows decreases in carbonic anhydrase 1 (*Car1*), solute carrier family 9 (sodium/hydrogen exchanger), member 2 (*Slc9a2*) and 3 (*Slc9a3*) mRNA abundance in *G6del* as compared to *Control* colon (n=7 in each group).

DISCUSSION

GATA4, 5, and 6 are members of an evolutionarily conserved zinc finger transcription factor family that play key roles in proliferation, differentiation and gene regulation in multiple organs,[3] but their roles in the gastrointestinal system are only beginning to be elucidated. GATA4 is expressed in the proximal 85% of the small intestine,[5, 17] is absent from distal ileum and colon, and regulates jejunal-ileal differences in absorptive enterocyte gene expression and function.[4-6] GATA6 is co-expressed with GATA4 in the proximal small intestine, but is also expressed throughout the epithelium of the ileum and colon. In the ileum, conditional *Gata6* deletion results in a decrease in cellular proliferation, a decrease in Paneth and enteroendocrine cells, an increase in goblet cells, and changes in absorptive enterocyte gene expression (Chapter 5). The absorptive enterocyte genes altered by conditional *Gata6* deletion are different from those regulated by *Gata4*, and many of those up-regulated by conditional *Gata6* deletion are genes that are expressed normally in colon (Chapter 5). Here, we show in colon that conditional deletion of *Gata6* results in alterations in

proliferation, secretory cell differentiation, and colonocyte gene expression paralleling GATA6 regulation in the small intestine. However, whereas *Gata6* deletion in small intestine resulted in an increase in mucin-2 (MUC2)-positive goblet cells, a general loss of enteroendocrine cells, and an increase in the expression of multiple genes not normally expressed in small intestine, but highly expressed in colon, *Gata6* deletion in colon resulted in a decrease in MUC2-positive goblet cells, a redistribution (rather than generalized loss) of enteroendocrine cell subtypes, and a down-regulation of specific colonocyte genes. These data demonstrate that GATA6 is necessary for the maintenance of proliferation, differentiation and gene expression in the colon in vivo, and regulates some of these processes differently than in the small intestine.

Cellular proliferation is required for the continuous renewal of the intestinal epithelium. We show that intestinal deletion of *Gata6* results in a reduction in proliferation in both small intestine (Chapter 5) and colon (Figure 3). Wnt signaling, a key pathway that promotes proliferation in the mouse small intestine and colon,[14, 18, 19] was not reduced in either organ (Figure 4 and Chapter 5), suggesting that the reduction in proliferation induced by *Gata6* deletion occurs independently of Wnt signaling. Although the mechanism remains to be elucidate, these data indicate that GATA6 is necessary for the maintenance of cellular proliferation in colonic crypts, and is thus likely required for the normal recycling of the colonic epithelium.

In the present study, we show that conditional *Gata6* deletion results in a reduction in MUC2 gene expression in goblet cells, and that this reduction is more pronounced in the lower half of colonic crypts (Figure 5A-C). We also show that fully differentiated TFF3-positive goblet cells that normally localize to the apical surface and upper third of the crypts were only found at the apical surface in *G6del* colon (Figure 5D, E). These findings suggest a delayed maturation process and/or an increased migration rate of committed goblet cells. It has been previously shown that deletion of *Muc2* results in impaired goblet cell differentiation, and an increase in the cellular migration rate in the colon.[20] Furthermore, in vitro studies have shown that GATA factors are capable of activating the *Muc2* promoter.[21, 22] These data suggest that in the colon GATA6 regulates terminal differentiation and migration of goblet cells by activating MUC2 expression.

In contrast to the reduction in MUC2 expression in colon, conditional *Gata6* deletion results in an accumulation of MUC2-positive goblet-like cells in ileal crypts (Chapter 5). Deletion of *Klf4* results in impaired goblet cell differentiation in the colon, but has no effect on goblet cell differentiation in the small intestine,[16] demonstrating that goblet cell differentiation is regulated differently in colon and small intestine. Our findings demonstrate that GATA6 promotes MUC2 expression and goblet cell differentiation in the colon, whereas GATA6 inhibits MUC2 expression and other goblet cell features in secretory cells in the small intestine, emphasizing different regulation of goblet cell differentiation between the small and large intestine.

Haveri et all show that GATA6 is not expressed in colonic enteroendocrine cells in humans.[9] In contrast, we show here that GATA6 is expressed in enteroendocrine cells in the mouse colon (Figure 1) and that intestinal deletion of *Gata6* results in a redistribution in enteroendocrine sublineages (Figure 6 and 7). Enteroendocrine cells in the colon are thought to all originate from

PYY-producing endocrine progenitor cells[23] from which subpopulations of enteroendocrine cells arise. Chromogranin A (CHGA) is expressed in most subtypes of enteroendocrine cells and therefore serves as a useful marker for the total enteroendocrine lineage.[24, 25] Most endocrine cells continue to express PYY, but the serotonin/tachykinin/secretin-expressing enteroendocrine cell population rarely coexpresses PYY.[23, 26-28] Our data suggest that GATA6 is not required for enteroendocrine cell commitment in the mature mouse colon, but defines the terminal differentiation of specific enteroendocrine sublineages, promoting the expression of SCT, CCK and GLP1 and inhibiting the expression of PYY.

The colonic surface epithelium is mainly responsible for the concentration of fecal effluent by absorbing water and electrolytes. Absorption is facilitated by a large osmotic gradient established by enzymes and transporters that are expressed within the absorptive colonocytes. Here, we show that although conditional *Gata6* deletion has no effect on multiple colonocyte genes, including *Car2* and *Atp1b1*, the colonocyte markers *Car1*, *Slc9a2* and *Slc9a3* are significantly down-regulated (Figure 8). Carbonic anhydrases catalyze the conversion of carbonic acids to bicarbonate and protons,[29] whereas the sodium-hydrogen exchangers utilize the protons generated by CAR to drive the internalization of Na+ and the subsequent absorption of water across the apical surface. CAR2 expression remains unchanged after *Gata6* deletion, and it is thus likely able to compensate for the decrease in CAR1. SLC9A2 (also known as NHE2) is the predominant SLC9A in the mouse colon.[30] Deletion of *Slc9a2* does not result in a diarrhea phenotype or any apparent Na+-absorptive defect,[31] however it results in an up-regulation of SLC9A3 (also known as NHE3).[30] Mice that lack *Slc9a3* [32] or both *Slc9a2* and *Slc9a3* [33] display diarrhea, demonstrating a role of SLC9A3 in Na+ absorption, and suggesting that the up-regulation of SLC9A3 in *Slc9a2*-null mice compensates for the lack of SLC9A2. The significant down-regulation of both SLC9A2 and SLC9A3 by conditional *Gata6* deletion (Figure 8) could therefore result in a decrease in the efficiency of water absorption by colonocytes in these mice. These data indicate that GATA6 is necessary for the expression of specific colonocyte genes important for water absorption and is thus critical for normal colonic function.

Interestingly, our data indicate that GATA6 regulates the same genes differently in the ileum and colon. For example, *Car1* and *Slc9a2* are down-regulated in the colon (Figure 8), but up-regulated in the ileum (Chapter 5); *Car2* is not changed in the colon (Figure 8), but up-regulated in the ileum (Chapter 5); and *Slc9a3* is down-regulated in the colon (Figure 8), but not changed in the ileum (Chapter 5). Intestinal genes down-regulated by conditional *Gata6* deletion, such as sucrase isomaltase and *Slc5a12*, are not normally expressed in the colon, and were not up-regulated in the colon after *Gata6* deletion (data not shown). These data indicate that the same genes are regulated by the same transcription factor (GATA6) differently in different organs. Since GATA factors are known to form multiple complexes that individually activate or repress target genes,[34] we hypothesize that GATA6 exists in multiple complexes, that the set of GATA6 complexes are different in small intestine and colon, and that specific information in the promoters of GATA6

target genes recruit different GATA6 complexes that ultimately regulate these genes differently in the small intestine and colon.

GRANTS

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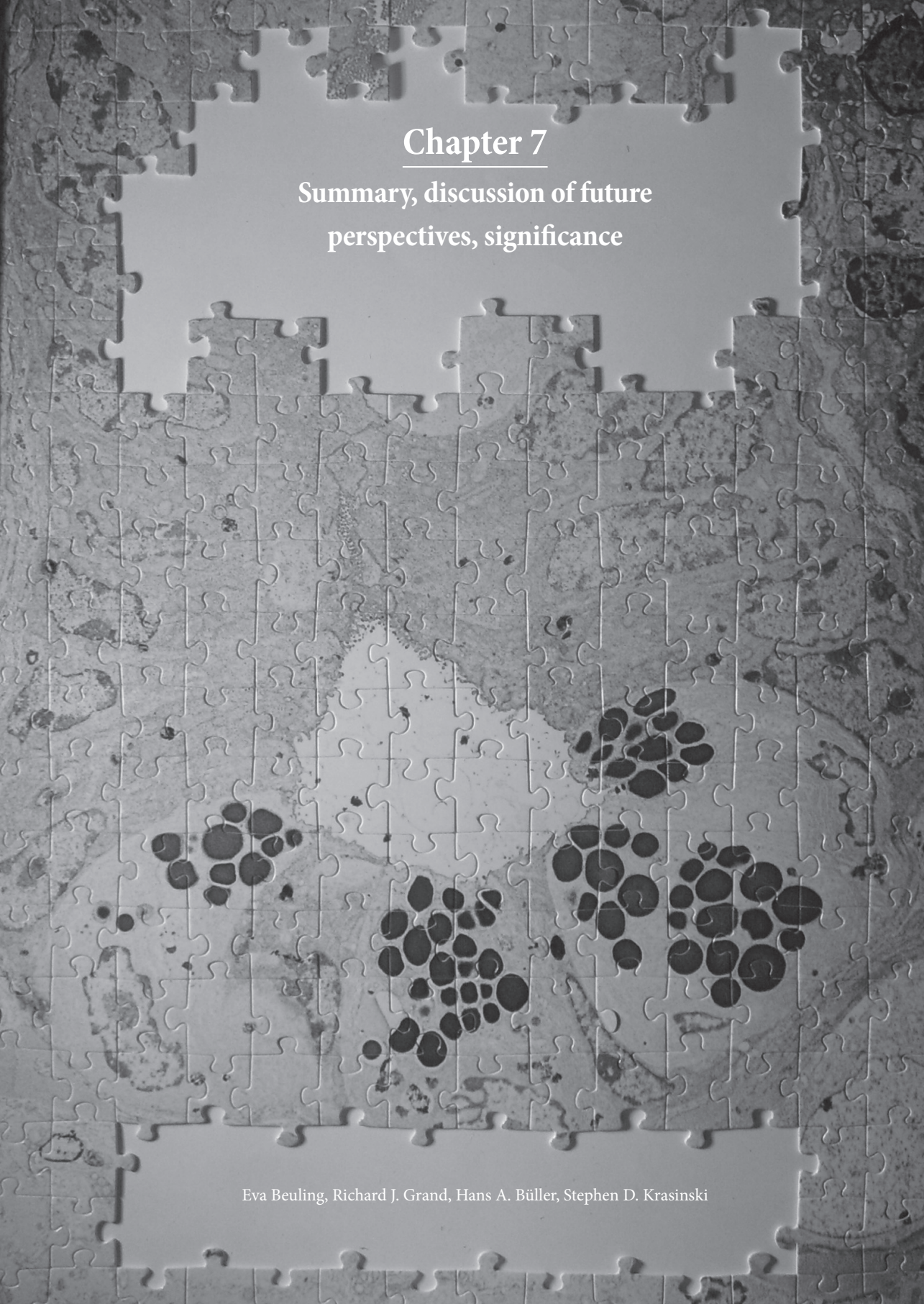
Chapter 7

Summary, discussion of future perspectives, significance



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SUMMARY

The mammalian intestine functions to absorb dietary nutrients and water, excrete waste materials and deny access to non-desirable substances.[1] To facilitate these specified and diverse functions, the lumen of the intestine is lined by a highly differentiated epithelium that is comprised of a monolayer of specialized columnar cells that are organized in a well-defined fashion.[2] The epithelial organization, with differences in cell distribution and protein expression along the cephalocaudal axis, is established during development and maintained throughout adulthood under the control of evolutionarily conserved mechanisms.[3] The *Hox* transcription factors are essential for the early morphogenesis of certain gut regions during early embryonic patterning, whereas the *ParaHox* gene cluster, that includes pancreatic-duodenum-homeobox 1 (*Pdx1*) and caudal type homeobox (*Cdx*) genes, plays critical roles in cephalocaudal endoderm patterning. During the cytodifferentiation of the intestinal epithelium, multiple molecular signaling pathways, such as canonical Wnt, Notch and Hedgehog, cooperatively establish and maintain throughout adulthood a functional intestinal epithelial structure by tightly regulating cellular proliferation, differentiation, migration and cell death.[4] Cephalocaudal differences in cell distribution and gene expression within the intestine that are established during intestinal patterning change to correlate with the functional needs of the intestine in different phases of development, and reach a stable state that is maintained throughout adulthood. Transcription factors, such as hepatocyte nuclear factor 1α (HNF1α) and GATA4, regulate the expression of intestinal genes that encode proteins involved in terminal digestion and absorption of nutrients in the mature small intestine.[4, 5] More specifically, GATA4 is essential for the maintenance of jejunal-ileal identities in the adult mouse small intestine.[4] This dissertation investigates the fundamental roles of GATA factors and their co-regulators in the underlying mechanisms that regulate proliferation, differentiation, cell commitment and gene expression on the cephalocaudal axis in the development and maintenance of the intestinal epithelium.

Lactase-phlorizin hydrolase (LPH), fatty acid binding protein 1 (FABP1), and sucrase-isomaltase (SI) are intestinal proteins important for the digestion and absorption of nutrients during different stages of development, and are established markers for the transitions that occur in intestinal development. [6-15] In rodents, LPH and FABP1 are first detected at the beginning of cytodifferentation, continue to be expressed at high levels during the suckling period, and decline during the weaning transition. In contrast, SI is undetectable before weaning and increases to adult levels during the weaning transition. Promoter studies of these intestinal genes have revealed binding sites for GATA and HNF1 transcription factors in close proximity to the transcriptional start site, and subsequent in vitro and cell culture assays have implicated these transcription factors as regulators of these intestinal genes.[16-34] Whereas in vivo mouse models have previously revealed that both GATA4 and HNF1α are absolutely required for the expression of *Lph* and *Fabp1* in the mature small intestine,[4, 5] the experiments described in **Chapter 2** demonstrate that GATA4 and HNF1α are dispensable or only partially required for the expression

of these intestinal genes before weaning. GATA4 is dispensable for *Lph* gene expression during both cytodifferentiation and suckling, whereas HNF1α is required for ~50% of *Lph* mRNA abundance during these same time intervals. GATA4 and HNF1α are both required for ~50% of *Fabp1* mRNA abundance during cytodifferentiation and suckling, but are both dispensable for *Si* gene expression at any time during development. Although at high protein levels during cytodifferentiation, during the suckling period GATA4 and HNF1α protein levels display a dramatic reduction, despite high levels of mRNA without a reduction in their requirement for the expression of *Lph* and *Fabp1* as compared to the cytodifferentation. Furthermore, neither GATA4 nor HNF1α mediates the glucocorticoid-induced precocious maturation of the intestine, but rather are downstream targets of this process. Together these findings show that specific intestinal genes have differential requirements for GATA4 and HNF1α that are dependent on the developmental time-frame in which they are expressed.

Friend of GATA (FOG) cofactors have been shown to mediate GATA4 function in cardiogenesis, gonadal differentiation and gastric epithelial development.[35-39] In the mature jejunum, GATA4 activates the expression of specific genes that are normally not expressed in the ileum (including *Lph* and *Fabp1*) and represses the expression of genes normally absent in jejunum but expressed in the ileum (including apical sodium-dependent bile acid transporter (*Asbt*) and ileal lipid binding protein (*Ilbp*)).[4, 40] Furthermore, GATA4 maintains the differences between jejunum and ileum in expression levels of the enteroendocrine cell lineage markers cholecystokinin (*Cck*) and peptide YY (*Pyy*), the secretory progenitor and goblet cell marker mouse atonal homolog 1 (*Math1*), and the goblet cell marker mucin 2 (*Muc2*).[4] The findings described in **Chapter 3** demonstrate that the GATA4-FOG interaction contributes to the repression of the expression of the ileum-specific absorptive enterocyte genes *Asbt* and *Ilbp* in the mature mouse jejunum, whereas this interaction is dispensable for the expression of *Lph*, *Fabp1*, *Cck*, *Pyy*, *Muc2* and *Math1*. These findings indicate that, within the mature small intestine, GATA4-FOG interactions contribute specifically and selectively to the repression function of GATA4 in absorptive enterocyte gene expression. *Fog1* mRNA displays a proximal-distal pattern that parallels that of *Gata4*, and FOG1 protein is co-expressed with GATA4 in intestinal epithelial cells, implicating FOG1 as the likely mediator of GATA4 function in the mature small intestine.

GATA4 determines jejunal-ileal identities in absorptive enterocyte gene expression in the adult mouse small intestine. One specific function of GATA4 is restricting the expression of ASBT, the rate-limiting transporter for bile acid absorption in the small intestine, to the distal small intestine by repressing the expression of ASBT in the proximal small intestine.[4] Ileal diseases and resections result in bile acid malabsorption due to loss of intestinal bile acid transport capacity.[41-43] The results in **Chapter 4** demonstrate that reduction of GATA4 activity results in an induction of ASBT and bile acid absorption in the proximal small intestine, and a depletion of luminal bile acids in the distal small intestine. The expression of the bile acid responsive genes *Ilbp*, organic solute transporter *(Ost)α and β*, and fibrobast growth factor *(Fgf15)* in the proximal small intestine generally increases, whereas their expression in the distal small generally decreases, correlating with

the shift from distal to proximal bile acid absorption. Bile acid absorption in the proximal small intestine does not result in alterations in fecal bile acid excretion, mRNA abundance of hepatic bile acid biosynthetic enzymes or bile acid pool size, demonstrating that the bile acid homeostatic machinery can adequately accommodate bile acids absorbed from the proximal small intestine. However, proximal bile acid absorption leads to tauro-β-muricholate enrichment of the bile acid pool due to a relative increase in tauro-β-muricholate uptake by the small intestine. Furthermore, the experiments show that ileocecal resection in mice[44] results in a marked increase of fecal bile acid excretion, a reduction of the bile acid pool, and a compensatory up-regulation of the expression of hepatic cholesterol 7-hydroxylase (*Cyp7a1*) mRNA, hallmarks of bile acid malabsorption reported in humans[41] and rats[45] after ileal resection. The induction of ASBT and bile acid absorption in the proximal small intestine following reduction of GATA4 activity corrects the bile acid malabsorpion associated with ileocecal resection in mice. Together these results demonstrate that reduction of GATA4 activity in the small intestine results in an induction of bile acid absorption in proximal small intestine that is sufficient to correct bile acid malabsorption associated with ileocecal resection in mice, without causing major alterations in bile acid homeostasis.

GATA4 and GATA6 are both expressed in the adult mouse small intestine.[4, 17, 46, 47] GATA4 is expressed at high levels throughout the small intestine, with the exception of the distal ileum, where it is undetectable, whereas GATA6 is expressed evenly throughout the small intestine.[4, 30] The experiments described in **Chapter 5** reveal that GATA6 is expressed in all differentiated and proliferating cells in the mature mouse small intestinal epithelium. In the mature distal ileum, GATA6 promotes proliferation and villus growth, possibly through a mechanism parallel to or in a co-regulatory fashion with the tumor suppressor gene p53 and myelocytomatosis oncogene (c-MYC). Furthermore, GATA6 promotes Paneth differentiation and enteroendocrine cell commitment likely by repressing SAM pointed domain containing ets transcription factor (SPDEF), and activating delta-like 1 (DLL1) and neurogenin 3 (NGN3) in the secretory progenitors in the mature ileum. In the absence of GATA6, secretory progenitors default to a goblet-like cell fate. In addition, GATA6 regulates the expression of specific intestinal genes within the absorptive enterocytes in the mature ileum. In the mature jejunum, intestinal *Gata6* deletion results in an increase in Paneth cells and the expression of Paneth cell markers, possibly as compensatory response to the loss of Paneth cells in the ileum. Furthermore, in the mature jejunum GATA4 is redundant for most but not all of the functions of GATA6. Together, these findings demonstrate that GATA factors promote proliferation, are required for proper secretory progenitor cell commitment, and regulate the expression of specific absorptive enterocyte genes in the mature small intestine.

GATA6, but not GATA4, is expressed in the mature mouse[18, 48] and human[49] colonic epithelium. **Chapter 6** shows that GATA6 is expressed in all colonic epithelial cells and promotes proliferation in the mature mouse colon. GATA6 is required for the activation of the expression of the colonocyte markers solute carrier family 9 (sodium/hydrogen exchanger), member 1 and 2 (SLC9A1 and SLC9A2), and carbonic anhydrase (CAR)1, but is dispensable for the expression of

its family member CAR2 and other colonocyte markers, demonstrating GATA6 is required for the expression of specific colonocyte genes. Furthermore, GATA6 promotes MUC2 expression and the terminal differentiation of goblet cells, and defines the terminal differentiation of specific enteroendocrine sublineages. Together these results demonstrate that, in the mature mouse colon, GATA6 promotes proliferation and regulates the terminal differentiation of colonocytes, goblet cells and enteroendocrine cells.

DISCUSSION OF FUTURE PERSPECTIVES

Absorptive enterocyte genes are differentially regulated before and after weaning

The most important conclusion that can be drawn from the experiments described in **Chapter 2** is that although GATA4 and HNF1α are both indispensable for the expression of the absorptive enterocyte genes *Lph* and *Fabp1* in the small intestine after weaning, these transcription factors are dispensable or only partially required for the expression of *Lph* and *Fabp1* in the small intestine before weaning. This finding suggests that for the expression of the absorptive enterocyte genes *Lph* and *Fabp1* during development, differential or redundant mechanisms exist that are not present in adulthood. This knowledge is particularly important for pediatric gastroenterology, since it reveals that the molecular mechanisms underlying the regulation of the expression of essential proteins for the digestive and absorptive function of the small intestine change with the maturation of the small intestine. In the first period of life, when mammals are dependent on milk for their nutrition, the digestion and absorption of lactose and lipids from milk is critical for survival. It is therefore very plausible that the expression of essential proteins for the digestion and absorption of nutrients in milk, such as LPH and FABP1, is regulated by redundant mechanisms in the suckling period to ensure their expression during this time frame in development. The transcription factors GATA5, GATA6, HNF1β, and members of the caudal (CDX), HNF3, and CCAAT/enhancer binding protein (C/EBP) families have been implicated as activators of *Lph* and/or *Fabp1* gene transcription in vitro,[16, 17, 20, 23, 50-56] and are therefore candidates for the regulation of *Lph* and *Fabp1* gene expression before weaning. Individual and combined, inducible, intestine-specific deletion models for these transcription factors will provide valuable information on their requirement in the regulation of *Lph* and *Fabp1* gene expression before weaning. Since inducible, intestine-specific deletion models for GATA6 and GATA6 + GATA4 have already been established and verified (**Chapter 5**) it will be straightforward to determine the requirement for GATA6 and GATA6 + GATA4 in the regulation of *Lph* and *Fabp1* gene expression before weaning. However, if GATA6 or GATA6 + GATA4 are indispensable for *Lph* and *Fabp1* gene expression before weaning, this knowledge will not explain the differential regulation of *Lph* and *Fabp1* gene expression after weaning, since GATA6 is co-expressed with GATA4 and HNF1α in the mature small intestine, but is unable to compensate for their loss in the regulation of *Lph* and *Fabp1* gene expression after weaning. Transcription factors that are functional in the

small intestine before weaning but not after weaning are therefore more likely to be responsible for determining the differential regulation of *Lph* and *Fabp1* gene expression before and after weaning. An alternative approach to determine factors that are required for *Lph* and *Fabp1* gene expression before weaning is to identify DNA sequences in the regulatory regions of the *Lph* and *Fabp1* genes that bind proteins before weaning but not after weaning. DNase hypersensitivity assays can identify DNA sequences that are accessible to transcription factors because they display increased sensitivity to digestion with DNaseI.[57] These so-called DNase hypersensitive sites are often located in the recognition sites for transcription factors, including promoters and enhancers. Comparing the DNAse hypersensitivity sites in the regulatory regions of the *Lph* and *Fabp1* genes in preweaning with postweaning epithelial cell nuclear extracts may lead to the identification of regions in the *Lph* and *Fabp1* genes that bind proteins before weaning but not after weaning. DNA sequence analysis software can be used to identify transcription factors that potentially bind to these regions, and chromatin immunoprecipitation (ChIP) assays can subsequently determine if the identified proteins indeed bind to these sites. DNAse hypersensitivity sites that bind GATA4 and HNF1α in the postweaning extracts can serve as positive controls. However, this is only true under the assumption that GATA4 and HNF1α regulate *Lph* and *Fabp1* expression in the adult by directly binding to their enhancers within the absorptive enterocytes, which is very likely, but as discussed later (see "Regulation of absorptive enterocyte genes by GATA4 and GATA6 through a mechanism that occurs within the absorptive enterocytes") not yet unequivocally determined. In contrast to transcription factor deletion models that use potential transcription factors as a starting point. Defining DNase hypersensitivity sites uses the genes of interest as a starting point and will therefore allow the identification of proteins that are not previously implicated as regulatory transcription factors in cell culture assays. Inducible, intestine-specific deletion models for the transcription factors identified by this approach can be used to confirm the in vivo requirement of these factors for the regulation of *Lph* and *Fabp1* gene expression before weaning. Together these experiments will provide essential information on the mechanisms that are responsible for the differential regulation of the expression of proteins essential for the absorptive and digestive function of the small intestine before and after weaning.

Co-factors contribute specifically to the repression function of GATA4 in the mature small intestine

The experiments described in **Chapter 3** suggest that within the mature mouse jejunum, GATA4-friend of GATA (FOG) interactions contribute specifically to the repression function of GATA4 in ileal-specific absorptive enterocyte gene expression. Furthermore, they show that *Fog1* mRNA displays a proximal-distal expression pattern that parallels that of *Gata4*, and FOG1 protein is co-expressed with GATA4 in intestinal epithelial cells, implicating FOG1 as the likely mediator of GATA4 function in the mature small intestine. To test the hypothesis that FOG1 contributes to the repression function of GATA4, we established a mouse line in which FOG1 is specifically deleted in the mature small intestinal epithelium by crossing the previously established FOG1

deletion model[36] with the Villin*Cre*ER^{T2} transgene used in all studies described in this dissertation. FOG1 mRNA and protein deletion in the mature jejunum in this model was confirmed by real time and Western blot analyses. However, no differences in *Asbt*, *Ilbp*, *Lph* and *Fabp1* mRNA abundances between FOG1 null and control jejunum were detected, demonstrating that FOG1 neither mediates the repression of *Asbt* and *Ilbp* nor the activation of *Lph* and *Fabp1* by GATA4 in the mature mouse jejunum. Since the mutation in GATA4 in the *Gata4* knockin model used in the study described in **Chapter 3** also disrupts the binding between GATA4 and FOG2 and the experiments described in **Chapter 3** show that *Fog2* mRNA is expressed, although at low levels, in the small intestine, we hypothesized that FOG2 is the FOG cofactor that mediates the repression function of GATA4 in the mature mouse jejunum. To test this hypothesis we established a model in which FOG2 would be specifically deleted in the mature small intestinal epithelium, by crossing the previously established FOG2 deletion model [38] with the Villin*Cre*ER^{T2} transgene. However, with this model we were unable to delete *Fog2* mRNA in the mature jejunum, suggesting that FOG2 is not expressed in the epithelium of the small intestine. A model in which the *LacZ* gene is expressed under the control of the *Fog2* promoter[58] showed β-galactosidase staining in the intestinal mesenchyme and not in the epithelium during development (Unpublished data, kindly shared with us by Dr. Sergei G. Tevosian and reported here with his permission.), demonstrating that FOG2 is expressed in the mesenchyme and not in the epithelium in the small intestine during development. In addition to the fact that we were unable to delete *Fog2* mRNA in the mature small intestine, this result suggests that FOG2 is not expressed in the mature small intestinal epithelium and therefore is not a candidate to mediate GATA4 function therein. Altogether these findings indicate that neither FOG1 nor FOG2 mediates GATA4 function in the mature small intestine, suggesting that the point mutation made in the N-terminal zinc finger of GATA4 in the *Gata4*^{ki} model reduces the repression capabilities of GATA4 in other ways than by disrupting the binding with FOG1 and/or FOG2. To test the hypothesis that GATA4 binds other (FOG-like) proteins that mediate its function, an approach that defines protein complexes that GATA4 forms in vivo could be utilized. Such an approach was used to define the protein complexes GATA1 forms for activating and repressing genes within erythroid cells.[59] A mouse line that expresses GATA4 tagged with a small peptide (Avi-tag) that is efficiently biotinylated by the bacterial BirA biotin ligase,[60] crossed with the previously established mouse strain that expresses the BirA gene ubiquitously,[61] will create a mouse line that expresses biotinylated GATA4. Biotinylation of GATA4 in vivo will allow efficient pull down of GATA4 protein complexes from intestinal epithelial nuclear extracts with streptavidin beads. Subsequent mass spectrometry can be utilized to identify the proteins pulled down with GATA4. Together these experiments will provide essential information on the different protein complexes that GATA4 forms in vivo and may lead to the identification of proteins that interact with GATA4 to define the activation versus repression function of GATA4.

Reduction of GATA4 activity in the mature small intestine as therapeutic intervention to induce bile acid absorption in the proximal small intestine after loss of ileal function

The major finding in the study described in **Chapter 4** is that conditional *Gata4* deletion induces *Asbt* expression and absorption of bile acids in the proximal small intestine that is sufficient to correct bile acid malabsorption in a mouse ileal resection model. This finding indicates that reduction of GATA4 activity in the small intestine is a possible therapeutic approach for correcting bile acid malabsorption associated with loss of ileal function. Several major questions will need to be addressed before these findings could be translated into a viable treatment. First, a non-genetic method for reducing GATA4 activity would have to be developed and validated for use in humans. Recently, a new approach was developed in which non-pathogenic bacteria were used to produce interfering RNA within cells.[62] Following oral or intravenous administration, engineered bacteria are taken up by the target tissue and release interfering RNA, thereby triggering the specific silencing of genes via the RNA interference (RNAi) pathway. The non-pathogenic bacteria are subsequently degraded by the host cells without adverse effects. This approach has been successfully used to knockdown the Wnt pathway effector, β-catenin, in mouse intestinal epithelial cells[62] demonstrating proof-of-concept. A similar bacterial RNAi knockdown vector for *Gata4* could be developed and used as a non-genetic method for GATA4 activity reduction. The efficacy of this vector to knockdown *Gata4* and induce bile acid absorption in the proximal small intestine of mice could be used as an initial proof-of-principle and validation of this strategy. Secondly, it will be necessary to further investigate the spectrum of potential positive or negative side effects of GATA4 activity reduction on other functions of the small intestine. It has been shown that GATA4 is required for the jejunal function of cholesterol and lipid absorption.[40] Therefore a partial reduction of GATA4 activity or specific reduction of the repression function of GATA4 in the mature small intestine that is sufficient enough to correct bile acid malabsorption associated with loss of ileal function, but minimizes potential negative effects may be desirable. Furthermore, the experiments described in **Chapter 4** show that although induction of bile acid absorption in the proximal small intestine does not lead to major defects in bile acid homeostasis, it results in a tauro-β-muricholate (TBMC) enriched and subsequently more hydrophilic bile acid pool. Since TBMC is not expressed in human (chenodeoxycholate is the human analog of TBMC), and the hydrophobicity indices of bile acids differ between humans and rodents,[63] it is difficult to predict what would happen to the bile acid pool composition in human after induction of bile acid absorption in the proximal intestine. In humans, ursodeoxycholate (UDC) and taurocholate (TC) are the more hydrophilic bile acids, whereas chenodeoxycholate (CDC), deoxycholate (DC) and lithocholate (LC) are the more hydrophobic bile acids. Induction of bile acid absorption in the proximal small intestine in humans may result in a more hydrophilic bile acid pool because of a decrease in the fraction of DC in the pool due to reduced cecal and colonic bacterial dehydroxylation of bile acids. Furthermore, like TBMC in mice, UDCA is poorly absorbed by ASBT in humans. By extending the length of time that UDCA is exposed to the ASBT (by increasing

the proximal expression of ASBT), UDCA absorption and thereby its fraction in the pool may increase, also leading to a more hydrophilic bile acid pool. Considering the association of DC with gallstone disease and colon cancer[64, 65] and cytoprotective effects of UDCA,[66] such changes would have potential positive side effects. However, the effects of absorption of bile acids in the proximal small intestine on the bile acid pool composition in humans should be determined by the development of reduction of GATA4 activity in the small intestine as a clinical approach. Together these experiments will determine the potential of GATA4 activity reduction in the small intestine as possible clinical approach for correcting bile acid malabsorption associated with loss of ileal function.

GATA6 and GATA4 promote proliferation in the mature intestine

One of the major findings from the experiments described in **Chapters 5 and 6** is that intestinal deletion of *Gata6* or *Gata6* + *Gata4* results in a reduction of cellular proliferation in the mature small and large mouse intestine. These results suggest that GATA6 in the ileum and colon, and both GATA6 and GATA4 in the jejunum promote cellular proliferation and may play a role in increasing proliferation during the adaptive response after loss of functional epithelial surface and/or uncontrolled polyp and tumor formation. Intestinal resection and radiation models have been used to study the adaptive response of the intestinal epithelium after loss of functional surface.[44, 67, 68] These studies have shown that loss or damage of functional epithelial surface results in epithelial hyperplasia of the remnant small intestine characterized by increases in proliferation of crypt epithelium leading to enhanced crypt depth, villus height, microvillus surface area, and functional absorptive capacity per unit length of intestine. Gastrointestinal secretions, luminal nutrients, and mesenchymal, neuronal and humoral factors have been implicated in the regulation of the adaptive response.[69-77] However, the exact mechanisms that underlie the increase in proliferation during the adaptive response remain to be elucidated. To test the hypothesis that GATA6 and GATA4 play a role in increasing proliferation during the adaptive response, the previously established inducible, intestine-specific *Gata6* + *Gata4* deletion model (**Chapter 5**) can be utilized. Comparison of the parameters of adaptive response, such as increased proliferation, enhanced crypt depth and villus height, between intestinal *Gata6* + *Gata4* null and control mice after they have undergone ileocecal resection (ICR)[44] as described in **Chapter 4** will determine the involvement of GATA6 and GATA4 in the regulation of the adaptive response after ICR. When increased proliferation becomes uncontrolled this can lead to undesirable polyp and tumor formation. Recently, increased expression of GATA6 or GATA6 + GATA4 has been correlated with neoplastic human gastrointestinal mucosa,[49] implicating GATA factors in the development of neoplastic lesions in the intestinal epithelium in humans. A model in which GATA6 and/or GATA4 are over-expressed in the mature mouse intestinal epithelium can be utilized to determine if increased expression of GATA6 and/or GATA4 results in an increase in proliferation and formation of neoplastic lesions in the intestinal epithelium. To over-express GATA factors specifically in the mature intestinal epithelium the previously generated pRosa26-DEST vector can be

used.[78] With the use of this vector, a mouse model can be established that allows cre-mediated expression of GATA6 or GATA4 cDNA constructs. The Rosa26 locus is ubiquitously expressed in adult mice. The insertion of a lox-STOP-lox cassette between the splice acceptor site and GATA6 or GATA4 cDNA in the pRosa26-DEST vector places the expression of the cloned fragment under the control of Cre recombinase. Crossing this mouse line with mice carrying the Villin*Cre*ER^{T2} transgene will create a mouse model in which GATA6 or GATA4 will be over-expressed in all intestinal epithelial cells upon tamoxifen treatment that can be used to test the effect of increased expression of GATA factors on proliferation and polyp and tumor formation. Together, the proposed experiments will determine the involvement of GATA6 and/or GATA4 in the regulation of increased proliferation during the adaptive response to loss of functional absorptive epithelium and the increased proliferation leading to polyp and tumor formation.

GATA4 and GATA6 are required for Paneth cell differentiation in the mature small intestine

Another important finding from the experiments described in **Chapter 5** is that intestinal deletion of *Gata6* results in a loss of Paneth cells in the ileum that coincides with a marked reduction in the expression of antimicrobial peptides normally produced by Paneth cells such as the alpha-defensins (also known as defensin related cryptdins), suggesting that GATA6 promotes Paneth cell differentiation in the mature mouse ileum. Furthermore, the experiments described in **Chapter 5** demonstrate that intestinal deletion of *Gata6* results in an increase in the number of Paneth cells and an increase in the expression of alpha-defensins in the jejunum, possibly as compensatory response to the loss of Paneth cells in the ileum. Intestinal deletion of *Gata4* in addition to intestinal deletion of *Gata6* results in a loss of Paneth cells in the jejunum as well as the ileum, indicating GATA4 is redundant for GATA6 in promoting Paneth cell differentiation in the jejunum and suggesting GATA4 is required for the compensatory increase of Paneth cells and the expression of alpha-defensins in the jejunum after intestinal *Gata6* deletion. In humans, a reduction in the expression of Paneth cell alpha-defensins in the ileum has been associated with ileal Crohn's disease, whereas Paneth cell metaplasia in the colon is commonly found in both ulcerative colitis and colonic Crohn's disease.[79, 80] Furthermore, it has been implied that regeneration and repair of affected mucosa in inflammatory bowel disease may be the most potent stimuli for causing colonic Paneth cell metaplasia.[79] Since the experiments in **Chapter 5 and 6** indicate that GATA6 and/or GATA4 promote Paneth cell differentiation in the small intestine as well as proliferation (necessary for regeneration and repair) in the small and large intestine, it will be interesting to investigate if the reduction in the expression of alpha-defensins, associated with ileal Crohn's disease, and the Paneth cell metaplasia, associated with ulcerative colitis and colonic Crohn's disease, correlate with alternations in the expression of GATA factors in human tissue. Furthermore, a model in which GATA6 and/or GATA4 are over-expressed as described in the previous paragraph could be used to determine if an increase in the intestinal expression of GATA factors leads to the expression of alpha-defensins and/or Paneth cell metaplasia in the mature

mouse colon. Together these experiments will provide information on the potential involvement of GATA factors in Crohn's disease and/or ulcerative colitis.

Regulation of absorptive enterocyte genes by GATA4 and GATA6 through a mechanism that occurs within the absorptive enterocytes

GATA6 and GATA4 are both expressed in differentiated absorptive enterocytes on the villi, but also in crypt epithelial cells ([4] and **Chapter 5**). It is therefore ambiguous whether these GATA factors regulate the expression of absorptive enterocyte genes by a process that occurs within absorptive enterocytes on villi, and/or that takes place early in the cell commitment and differentiation program in crypt progenitor cells. In vitro and cell culture assays have shown that GATA factors bind to the enhancers of their target genes, and that this protein-DNA interaction is required for the regulation of the expression of these genes within differentiated cell lines.[16, 17, 20, 23] However, the cell commitment and differentiation process that absorptive enterocytes go through in vivo is not represented in these in vitro and cell culture assays. Experiments described in **Chapter 3** show that intestinal inactivation of *Gata4* in mice leads to expression of ASBT in absorptive enterocytes at the tip of the villi within 48 hours. Because of the 3-day crypt-to-villus tip cell migration time in mice, these results suggest that GATA4 inhibits the expression of ASBT within the absorptive enterocytes. Experiments described in **Chapter 5** show that after deletion of *Gata6* the absorptive enterocyte lineage commitment remains unchanged, while the expression of specific absorptive enterocyte genes is markedly up- or down-regulated. Taken together, these data suggest that GATA factors regulate the expression of absorptive enterocyte genes within the absorptive enterocytes. To unequivocally determine if GATA4 and GATA6 regulate the expression of absorptive enterocyte genes through a mechanism that takes place within the absorptive enterocytes rather than one that occurs in crypt progenitor cells, a model in which GATA4 and/or GATA6 are deleted specifically in differentiated absorptive enterocytes should be established. This could be realized by the development of a transgene that directs the expression of the inducible DNA recombinase *Cre*ER^{T2} specifically to the absorptive enterocytes. Since SI is specifically expressed in absorptive enterocytes, and GATA4 and GATA6 are dispensable for its expression in the mature jejunum (**Chapter 5**), the regulatory region of the *Si* gene would be a good candidate for the promoter part of the transgene. Crossing mice carrying the SI*Cre*ER^{T2} transgene with the previously established inducible GATA4, GATA6, and GATA6 + GATA4 deletion models will create inducible, absorptive enterocyte specific GATA4 and/or GATA6 models. With these models the hypothesis that GATA4 and GATA6 regulate absorptive enterocyte gene expression by a mechanism that takes place within the absorptive enterocytes can be tested.

Differential regulation of enterocyte genes in the small and large intestine by GATA factors

The research described in this dissertation demonstrates that the expression of specific enterocyte genes in the mature mouse intestine is differentially regulated by GATA4 and/or GATA6

dependent on the localization along the cephalocaudal axis. LPH is expressed in the jejunum, and is absent from the ileum,[81] whereas the expression of ASBT is restricted to the ileum.[42] Previous studies[4, 40] and the experiments described in **Chapter 4** show that GATA4 is indispensible for the regulation of specific absorptive enterocyte genes, including the repression of *Asbt* and the activation of *Lph* in the mature jejunum. These findings demonstrate that the exclusion of GATA4 expression from the distal small intestine serves as a mechanism to establish differences in gene expression between jejunum and ileum. Furthermore, the experiments show that although GATA6 is co-expressed with GATA4 in the mature jejunum, GATA6 is unable to compensate for the loss of GATA4 in the repression of *Asbt* and the activation of *Lph* gene expression. These data indicate that these particular GATA4 target genes contain features that specify their explicit regulation by GATA4 and exclude regulation by GATA6. SI is expressed throughout the small intestine.[6] The experiments described in **Chapter 2** demonstrate that, although capable of activating *Si* expression in cell culture and in vitro models, GATA4 is dispensable for the expression of *Si* in vivo at any time during development. These results suggest that other factors are required for the expression of *Si* in the mouse small intestine. Since mammals are mainly dependent on solid food for nutrition after weaning, it is not unlikely that the expression of proteins important for the digestion and absorption of nutrients from solid food is regulated by redundant mechanisms to ensure their expression. The experiments described in **Chapter 5** demonstrate that GATA6 is required for the activation of *Si* expression in the mature mouse ileum, whereas both GATA6 and GATA4 are dispensable for *Si* expression in the mature mouse jejunum. Very similar results were found for the regulation of *Slc2a1* gene expression; GATA6 is required for the repression of *Slc2a1* in the mature ileum, whereas both GATA6 and GATA4 are dispensable for the expression of *Slc2a1* in the mature jejunum. These findings show that GATA6 activates *Si* expression and represses *Slc2a1* expression in the mature mouse ileum, and suggest that factors other than GATA4 and GATA6 regulate the expression of *Si* and *Slc2a1* in the mature mouse jejunum. The experiments described in **Chapter 5 and 6** demonstrate that the colonocyte marker CAR1 is not expressed or at very low levels in the mature small intestine, whereas it is highly expressed in the colonic epithelium. GATA6 differentially regulates the expression of *Car1* in the ileum and the colon. GATA6 represses the expression of *Car1* in the ileum (**Chapter 5**), whereas in the colon GATA6 activates the expression of *Car1* (**Chapter 6**). Furthermore, GATA6 is dispensable for the expression of *Car1* in the jejunum, whereas both GATA4 and GATA6 are required for the repression of *Car1* expression in this region of the intestine (**Chapter 5**). Altogether these findings demonstrate at least 4 different ways by which GATA factors regulate the expression of specific enterocyte genes. The expression of specific enterocyte genes is (a) specifically regulated by GATA4 in the jejunum, although GATA6 is co-expressed, (b) regulated by GATA6 in the ileum, and regulated by both GATA6 and GATA4 in the jejunum, (c) regulated by GATA6 in the ileum, and regulated by other factors than GATA6 and GATA4 in the jejunum, and (d) activated by GATA6 in the colon, repressed by GATA6 in the ileum, and repressed by both GATA6 and GATA4 in the jejunum. The combination of defining DNA hypersensitivity sites in the regulatory regions of the

specific enterocyte genes as described in paragraph Absorptive enterocyte genes are differentially regulated before and after weaning using epithelial nuclear extracts from the jejunum, ileum and colon, together with determining the protein complexes formed by GATA4 and/or GATA6 in vivo as described in paragraph Co-factors contribute specifically to the repression function of GATA4 in the mature small intestine will help to define the mechanisms that are responsible for the differential regulation of specific enterocyte genes by GATA4 and GATA6 in the mature intestine.

SIGNIFICANCE

The mammalian intestinal epithelium requires a well coordinated expression of genes and distribution of cell types along the length of the intestine to efficiently digest and absorb nutrients. These processes, however, are easily disrupted by disease, congenital deviations or inescapable resections. To eventually develop strategies to regenerate lost or deficient intestinal function when gastrointestinal processes go awry, it is essential to understand the molecular events that are responsible for the development and maintenance of a normally functioning intestine. This dissertation describes the research that discovered essential roles for the evolutionarily conserved GATA family of transcription factors in the regulation of proliferation, differentiation, cell commitment and gene expression along the cephalocaudal axis in the development and maintenance of the normally functioning intestinal epithelium. GATA4, a transcription factor that is expressed in the proximal small intestine but not in the distal ileum, determines jejunal-ileal absorptive identities by activating and repressing specific absorptive enterocyte genes in the proximal small intestine. GATA4 and GATA6 are co-expressed in villus and crypt cells of the proximal small intestine, where they promote proliferation, Paneth cell differentiation, and enteroendocrine cell commitment. Furthermore, GATA4 and GATA6 differentially regulate the expression of absorptive enterocyte genes dependent on the expression patterns of these genes on the cephalocaudal axis. In contrast to GATA4, GATA6 is expressed in distal small intestine and colon where it promotes proliferation and regulates terminal differentiation and gene expression. Future studies proposed in this dissertation will utilize a series of new in vivo models to define the mechanisms by which GATA4 and GATA6 mediate proliferation and differentiation processes in the intestine. The results of these studies will increase our understanding of the molecular control mechanisms that underlie intestinal proliferation, gene regulation, and cell fate specification, creating a critical foundation for understanding current, and developing future, therapeutic interventions for regenerating lost or deficient intestinal function. These studies may also provide critical new insight into mechanisms of inflammatory bowel disease, cancer progression and intestinal adaptation.

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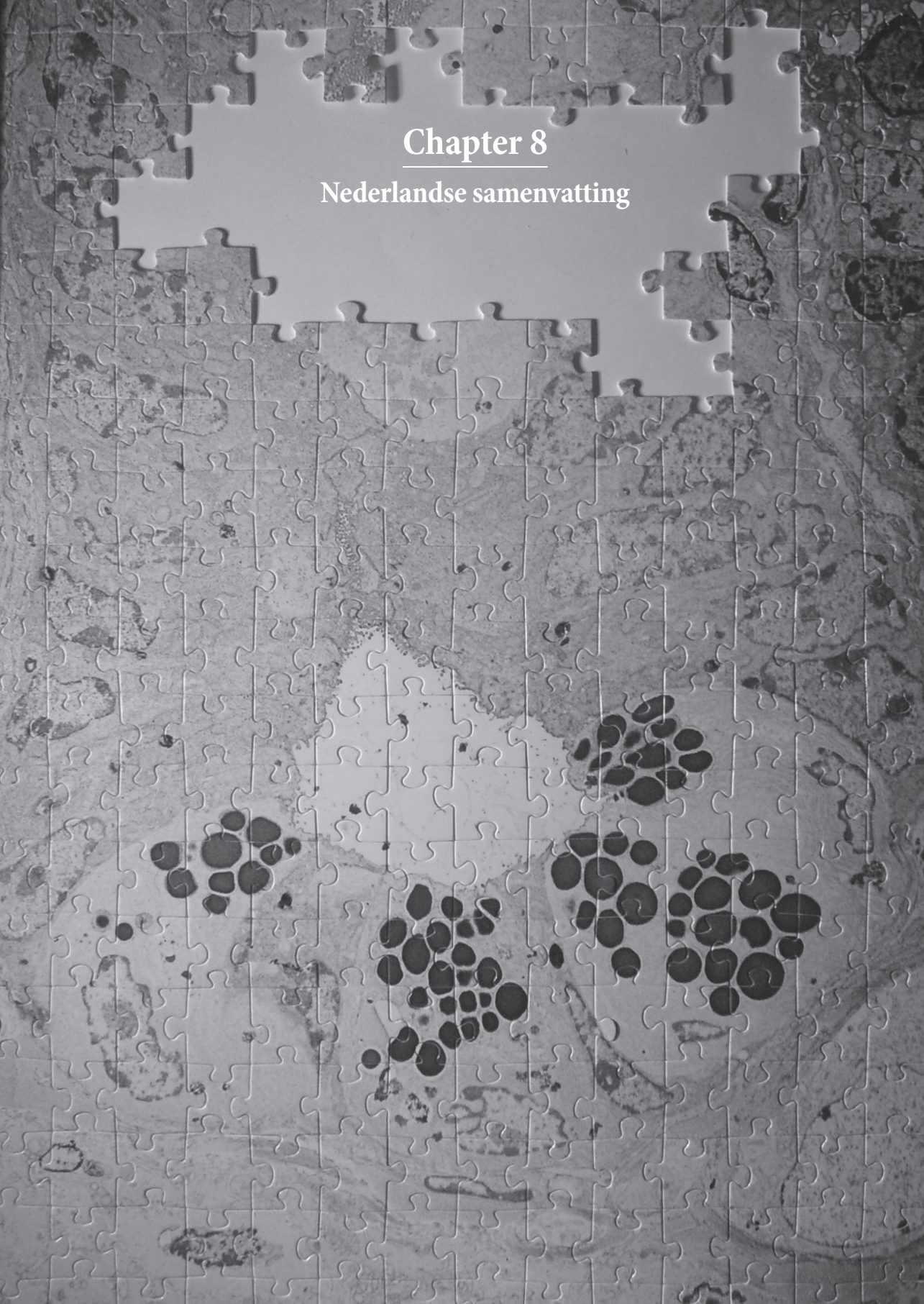
Chapter 8

Nederlandse samenvatting



INLEIDING IN DE ONTWIKKELING EN DIFFERENTIATIE VAN HET DARMEPITHEEL

De darm is verantwoordelijk voor de opname van voedingsstoffen en water, het blokkeren van de toegang voor schadelijke stoffen, en uiteindelijk het uitscheiden van afvalstoffen. Om deze specifieke en diverse functies uit te kunnen voeren is de binnenwand van de darm over zijn gehele lengte bekleed met een epitheellaag, die opgebouwd is uit verschillende cellen met ieder een eigen specifieke functie. De belangrijkste celtypes in de epitheellaag van de dunne darm zijn: (1) de enterocyten; cilindervormige trilhaar-epitheelcellen die verantwoordelijk zijn voor de vertering, opname, transport en uitscheiding van stoffen, (2) de slijmbekercellen; slijmproducerende en bekervormige epitheelcellen die verantwoordelijk zijn voor de productie van een beschermende slijmlaag tegen mechanische en chemische schade, (3) de entero-endocriene cellen; hormoonproducerende epitheelcellen die processen aansturen zoals het uitscheiden van maagsappen door de maag, het ledigen van de maag, het uitscheiden van verteringssappen door de alvleesklier en zorgdragen voor de peristaltische bewegingen in de darm en (4) de cellen van Paneth; antimicrobe stoffen uitscheidende epitheelcellen die een rol spelen in het immuunsysteem van de darm. In de epitheellaag van de dikke darm komen enterocyten, slijmbekercellen en entero-endocriene cellen voor, maar geen cellen van Paneth. De darmwand bestaat uit een grote hoeveelheid vouwen om het oppervlak en zodoende het opnemend vermogen zo groot mogelijk te maken. In de dunne darm eindigen deze vouwen in vingervormige uitstulpingen, de zogenaamde villi, en bevinden zich in de gevouwen darmwand inhammen, de zogenaamde crypten, om het opnemende vermogen nog groter te maken. In de gevouwen darmwand van de dikke darm bevinden zich alleen crypten en geen villi. Alle epitheliale darmcellen maken eiwitten aan, zoals verteringsenzymen, pompen, transporteiwitten, slijm, hormonen en antimicrobe stoffen waarmee de cellen hun gespecialiseerde functies kunnen uitvoeren. De verdeling van de verschillend gespecialiseerde epitheelcellen langs de villus-crypt en de cephalocaudale assen van de darm wordt nauwkeurig aangestuurd, zodat de vertering en opname van voedingsstoffen en water, en het uitscheiden van afvalstoffen over de gehele lengte van de darm in een efficiënte volgorde plaats vinden. Deze structuur van het darmepitheel wordt in stand gehouden doordat stamcellen, die zich in de crypten bevinden, continu delende precursor cellen afgeven. Terwijl ze zich verplaatsen naar hun uiteindelijke locatie (villus voor enterocyten, slijmbekercellen en endocriene cellen en de cryptbasis voor de cellen van Paneth) differentiëren de dochtercellen van de precursor cellen, om zo uit te groeien tot de vier bovengenoemde types darmcellen. De villusepitheelcellen bereiken in drie à vier dagen de villustop, ondergaan geprogrammeerde celdood en exfoliëren in het darmlumen. De cellen van Paneth ontsnappen aan de opwaartse migratiestroom, verblijven in de cryptbasis en hebben een levensduur van drie tot zes weken.

De ontwikkeling en de levensduur van het darmepitheel wordt gereguleerd door evolutionair geconserveerde moleculaire mechanismen. Tijdens de embryonale ontwikkeling wordt de primitieve darm gevormd uit het endoderm en mesoderm, de binnenste en middelste kiembladen van

de drie embryonale kiembladen. De transcriptiefactoren familie die gecodeerd wordt door de *Hox* genen, speelt een belangrijke rol in de orgaanbepaling tijdens de vroege embryonale ontwikkeling. Deze familie van transcriptiefactoren reguleert de expressie van genen, die ervoor zorgen dat verschillende delen langs de cephalocaudale as van het embryo uitgroeien tot de juiste organen. Hiermee wordt de basis gelegd voor de verschillende delen waaruit het primitieve maagdarmkanaal bestaat; de voordarm, de middendarm en de einddarm. Later in de embryonale ontwikkeling ontstaan uit de voordarm de slokdarm, de maag en het proximale deel van het duodenum (eerste gedeelte van de dunne darm); uit de middendarm ontwikkelen zich het distale deel van het duodenum, het jejunum (middelste gedeelte van de dunne darm), het ileum (laatste gedeelte van de dunne darm), het proximale deel van de dikke darm en uit de einddarm het distale deel van de dikke darm. Transcriptiefactoren van de *Parahox* familie, "pancreatic and duodenal homeobox" (PDX) en "caudal type homeobox" (CDX), sturen deze ontwikkeling van de darm langs de cephalocaudale as aan. Tijdens dit proces wordt de darm ook aanzienlijk langer. Als de indeling van de darm in de verschillende onderdelen heeft plaatsgevonden, ondergaat het darmepitheel een van proximaal naar distaal verlopende transformatie. Ongedifferentieerd epitheel, bestaande uit platte cellen, transformeert naar gedifferentieerd epitheel met cilindervormige cellen. Gelijktijdig met deze cytodifferentiatie onstaat de villus-crypt structuur. Deze processen van cytodifferentiatie en villus-cryptformatie, en de instandhouding van de structuur van de epitheellaag, worden aangestuurd door moleculaire signaleringscascades, zoals de Wnt, Notch en Hedgehog signaleringscascades, die gezamenlijk proliferatie, differentiatie, migratie en geprogrammeerde celdood reguleren. Verschillen langs de cephalocaudale as van de darm in de distributie van de verschillende celtypes en eiwitten die tot expressie komen in de gedifferentieerde cellen, ontstaan tijdens de cytodifferentiatie in de embryonale ontwikkeling. De distributie van celtypes en eitwitexpressie veranderen om aan te sluiten bij de functionele behoefte van de darm tijdens verschillende fasen in de ontwikkeling van de darm en bereiken een stabiele situatie in de volgroeide darm. In de eerste postnatale fase, de periode van de zuigeling, is de distributie van cellen en de expressiepatronen van eiwitten in gedifferentieerde cellen in het darmepitheel ingericht voor een efficiënte vertering en opname van voedingstoffen uit moedermelk. Wanneer het voedingspatroon van de pasgeborene overgaat van melkvoeding naar vast eten (de "weaning" transitie), veranderen deze patronen in het darmepitheel, zodat de voedingsstoffen uit vast eten goed verteerd en opgenomen kunnen worden. Eerder *in vivo* onderzoek heeft aangetoond dat de transcriptiefactoren "hepatic nuclear factor 1 alpha" (HNF1α) en GATA4 de expressie van bepaalde darmspecifieke genen, die coderen voor eiwitten die betrokken zijn bij de vertering en opname van voedingsstoffen in de dunne darm, reguleren. GATA4 is verantwoordelijk voor de instandhouding van de verschillen in genexpressie tussen het jejunum en het ileum.

DE ROL VAN GATA TRANSCRIPTIEFACTOREN IN DE REGULATIE VAN DE ONTWIKKELING, DIFFERENTIATIE EN FUNCTIE VAN DE DARM

Er is al veel bekend is over de mechanismen, die verantwoordelijk zijn voor de ontwikkeling en de instandhouding van het darmepitheel. Toch is nog meer kennis nodig om interventies te ontwikkelen die de structuur en functie van de darm kunnen herstellen in het geval van ziekteprocessen in de darm. Het doel van het onderzoek dat beschreven wordt in dit proefschrift is het vaststellen van de rol van de GATA transcriptiefactoren in de moleculaire mechanismen die ten grondslag liggen aan de processen van proliferatie, differentiatie en genexpressie langs de cephalocaudale as van de darm tijdens de ontwikkeling en de instandhouding van functioneel darmepitheel.

GATA4 en HNF1α zijn gedeeltelijk verantwoordelijk voor de expressie van darmspecifieke genen tijdens de ontwikkeling van de dunne darm

Lactase-phlorizin hydrolase (LPH), fatty acid binding protein 1 (FABP1), en sucrase-isomaltase (SI) zijn darmeiwitten die tot expressie komen in enterocyten en belangrijk zijn voor de vertering en opname van voedingsstoffen. De expressiepatronen van deze eiwitten veranderen tijdens de verschillende fasen in de ontwikkeling van de darm. Deze tijdsafhankelijke kritische perioden zijn goede modellen voor het bestuderen van regulatie van genexpressie tijdens de ontwikkeling van de darm. Eerder onderzoek heeft aangetoond dat de transcriptie van de genen die coderen voor LPH (het *Lph* gen), FABP1 (het *Fabp1*gen) en SI (het *Si* gen) en de translatie van het messenger RNA (mRNA) naar eiwitten parallel lopen. In de prenatale dunne darm van knaagdieren komen de *Lph* en *Fabp1* genen voor het eerst tot expressie aan het begin van de cytodifferentiatie. De mate van genexpressie van *Lph* en *Fabp1* is maximaal tijdens de neonatale fase en neemt geleidelijk af tijdens de "weaning" periode tot de minimale expressie in de volgroeide darm bereikt is. In tegenstelling tot LPH en FABP1, komen het *Si* gen en het SI eiwit voor het eerst tot expressie aan het einde van de moedermelkfase, nemen geleidelijk toe tijdens de "weaning" periode en bereiken het hoogste niveau in de volledig ontwikkelde darm. Eerder *in vitro* onderzoek met gekweekte darmcellen heeft aangetoond dat de GATA en HNF1 transcriptiefactorenfamilies de transcriptie van de *Lph*, *Fabp1* en *Si* genen kunnen aansturen door zich te binden aan de bindingselementen die zich in deze genen bevinden. Verder is *in vivo* aangetoond, dat zowel GATA4 als HNF1α noodzakelijk zijn voor de transcriptie van de *Lph* and *Fabp1* genen in de volgroeide darm van de muis. De experimenten beschreven in **Hoofdstuk 2** laten zien dat GATA4 en HNF1α maar gedeeltelijk of helemaal niet verantwoordelijk zijn voor de expressie van darmspecifieke genen tijdens de ontwikkeling van de darm van de muis. GATA4 is niet essentieel voor de transcriptie van het *Lph* gen tijdens de cytodifferentiatie en de neonatale fase, terwijl HNF1α zorg draagt voor 50% van de mate van genexpressie van het *Lph* gen tijdens deze fasen in de ontwikkeling. GATA4 en HNF1α zijn verantwoordelijk voor 50% van de mate van genexpressie van het *Fabp1* gen tijdens de cytodifferentiatie en de neonatale fase, maar GATA4 en HNF1α zijn op geen enkel moment in de ontwikkeling noodzakelijk voor de expressie van het *Si* gen. Tijdens de cytodifferentiatie zijn

de eiwitspiegels van GATA4 en HNF1α hoog, maar deze spiegels nemen dramatisch af in de neonatale periode, terwijl de *Gata4* en *Hnf1α* mRNAspiegels daarentegen stabiel blijven. Ondanks het feit dat de eiwitspiegels van GATA4 en HNF1α dalen tijdens de neonatale periode, zijn deze transcriptiefactoren gedurende deze fase in gelijke mate verantwoordelijk voor de transcriptie van de *Lph* en *Fabp1* genen als tijdens de cytodifferentiatie. Verder laten de experimenten beschreven in **Hoofdstuk 2** zien, dat vroegtijdige ontwikkeling van de dunne darm die geïnduceerd wordt door glucocorticoiden ook plaatsvindt als het *Gata4* of het *Hnf1α* gen is uitgeschakeld. Deze resultaten betekenen dat de GATA4 en HNF1α eiwitten dit proces niet aansturen. Concluderend tonen deze bevindingen aan dat de expressie van darmspecifieke genen verschillend aangestuurd wordt door de transcriptiefactoren GATA4 en HNF1α, afhankelijk van het tijdstip in de ontwikkeling waarop ze tot expressie komen.

GATA4 remt genexpressie in de volgroeide proximale dunne darm door interactie aan te gaan met friend of GATA (FOG) co-factoren

Eerder onderzoek heeft uitgewezen dat "friend of GATA" (FOG) co-factoren de functie van GATA4 tijdens cardiogenese, geslachtsorgaandifferentiatie en de ontwikkeling van het maagepitheel beïnvloeden door een fysieke interactie aan te gaan met de zinkvinger aan de N-terminus kant van het GATA4 eiwit. GATA4 komt niet in de distale dunne darm, maar wel in de proximale dunne darm tot expressie. Eerder onderzoek heeft aangetoond dat GATA4 de transcriptie van darmgenen, die normaal alleen in het jejunum en niet in het ileum tot expressie komen (zoals *Lph* en *Fabp1*), activeert. Daarnaast remt GATA4 de transcriptie van darmgenen die niet in het jejunum maar wel in het ileum tot expressie komen (zoals *Asbt* en *Ilbp*). Ook houdt GATA4 de verschillen in de mate van genexpressie van de hormonen CCK en PYY in endocriene cellen, en de MATH1 en MUC2 eiwitten in slijmbekercellen en secretoire precursorcellen tussen het jejunum en ileum in stand. De bevindingen die beschreven worden in **Hoofdstuk 3** laten zien dat het verbreken van de fysieke verbinding tussen GATA4 en FOG co-factoren resulteert in de expressie van de ileum-specifieke enterocytgenen *Asbt* en *Ilbp* in het jejunum. Daarnaast laten de resultaten zien dat het verbreken van deze interactie geen invloed heeft op de genexpressie van *Lph*, *Fabp1*, *Cck*, *Pyy*, *Math1* en *Muc2*. Deze bevindingen suggereren dat de interactie tussen GATA4 en FOG co-factoren specifiek bijdraagt aan de inhiberende werking van GATA4 op de expressie van de ileum-specifieke enterocytgenen *Asbt* en *Ilbp* in het jejunum. Er bestaan twee FOG co-factoren, FOG1 en FOG2. In **Hoofdstuk 3** worden de expressiepatronen van deze twee FOG co-factoren genen en eiwitten in de dunne darm beschreven. Het expressiepatroon van het *Fog1* mRNA langs de cephalocaudale as van de darm loopt parallel aan het expressiepatroon van het *Gata4* mRNA. Het FOG1 eiwit komt samen met het GATA4 eiwit voor in darmepiteelcellen, terwijl het expressieniveau van het *Fog2* mRNA langs de hele lengte van de dunne darm laag is en het FOG2 eiwit zelfs ondetecteerbaar is. Deze resultaten suggereren, dat FOG1 de FOG co-factor is, die de inhiberende functie van GATA4 op de expressie van ileum-specifieke genen in de dunne darm medieert.

Vermindering van de activiteit van GATA4 in de volwassen dunne darm resulteert in de opname van galzuren in de proximale dunne darm

Eerder onderzoek heeft aangetoond dat GATA4 de verschillen in de expressie van enterocytgenen tussen het jejunum en ileum in de dunne darm van de volgroeide muis bepaalt. Eén specifieke functie van GATA4 zorgt dat de "apical sodium-dependent bile acid transporter" (ASBT), de transporter die verantwoordelijk is voor de opname van galzuren, alleen in het distale gedeelte van de dunne darm voorkomt. Dit bewerkstelligt GATA4 door de expressie van ASBT in het proximale deel van de dunne darm te remmen. Ziektes aan het ileum en resecties van dit distale gedeelte van de dunne darm leidden tot malabsorptie van galzuren, doordat de capaciteit van de dunne darm om galzuren op te nemen verloren gaat. De resultaten beschreven in **Hoofdstuk 4** laten zien dat reductie van de activiteit van GATA4 in de darm resulteert in de expressie van het *Asbt* mRNA en het ASBT eiwit, en dien ten gevolge de opname van galzuren in het proximale gedeelte van de dunne darm. De opname van galzuren in de proximale dunne darm zorgt ervoor dat er zich bijna geen galzuren meer in de darminhoud van de distale dunne darm bevinden. Door de opname van galzuren in de proximale dunne darm vindt er een stijging plaats van de mate van de mRNA expressie in de proximale dunne darm van de "ileal lipid binding protein" (*Ilbp*), "organic solute transporter alpha and beta)" (*Ostα* en *Ostβ*) en "fibrobast growth factor 15" (*Fgf15*) genen, waarvan bekend is dat hun expressie door galzuren gereguleerd wordt. De mate van expressie van deze genen verlaagt in de distale dunne darm door de verlaging in galzuuropname in de distale dunne darm. Ondanks het feit dat de opname van galzuren verplaatst van de distale dunne darm naar de proximale dunne darm nadat de activiteit van GATA4 is gereduceerd, blijft de galzuurhomeostase grotendeels intact. Het is opmerkelijk, dat de hoeveelheid galzuren die uitgescheiden worden in de ontlasting niet verandert, als galzuren in de proximale dunne darm worden opgenomen. Ook de mate van expressie van enzymen die verantwoordelijk zijn voor de synthese van galzuren in de lever blijft constant en de totale hoeveelheid galzuren in de lichaamspool blijft stabiel. Deze bevindingen laten zien dat de galzuurhomeostase adequaat kan omgaan met galzuren die door de proximale dunne darm worden opgenomen. Alhoewel de galzuurhomeostase grotendeels onveranderd blijft, leidt de opname van galzuren in de proximale dunne darm tot een verrijking van de hoeveelheid tauro-β-muricholisch galzuur in de lichaamspool. Dit is niet het resultaat van een verhoogde synthese van tauro-β-muricholisch galzuur door de lever, maar van een relatief verhoogde tauro-β-muricholisch galzuur opname van de dunne darm. Verder laten de experimenten beschreven in **Hoofdstuk 4** zien, dat ileocecale resectie in muizen resulteert in (1) een toename van de hoeveelheid galzuren die wordt uitgescheiden in de ontlasting, (2) een afname van de hoeveelheid galzuren in de lichaamspool, en (3) een compensatoire verhoging van de expressie van het *Cyp7a1* gen dat codeert voor cholesterol 7α-hydroxylase, het belangrijkste enzym in de synthese van galzuren in de lever. Dit zijn kenmerken van galzuurmalabsorptie na ileoectomie zoals deze in mensen en ratten zijn beschreven. De experimenten beschreven in **Hoofdstuk 4** laten zien, dat galzuurmalabsorptie, als gevolg van ileocecale resectie in muizen, wordt gecorrigeerd door expressie van ASBT eiwit en de opname van galzuren in het

proximale gedeelte van de dunne darm na GATA4 activiteitsverlaging. Deze opname van galzuren in de proximale dunne darm is toereikend om de galzuurmalabsorptie in muizen na ileocecale resectie te corrigeren, zonder dat daarbij grote veranderingen in de galzuurhomeostase teweeg worden gebracht.

GATA factoren stimuleren proliferatie en zijn verantwoordelijk voor de differentiatie van cellen in het epitheel van de dunne darm

GATA4 en GATA6 eiwitten komen beide tot expressie in de volgroeide dunne darm van de muis. GATA4 is in de gehele dunne darm aanwezig, met uitzondering van het distale deel, terwijl GATA6 in de totale dunne darm voorkomt. De experimenten die beschreven zijn in **Hoofdstuk 5** tonen aan dat het GATA6 eiwit voorkomt in alle gedifferentieerde en prolifererende cellen in het darmepitheel van de dunne darm in de volgroeide muis. GATA6 stimuleert proliferatie en villusgroei in het ileum. Het mechanisme dat ten grondslag ligt aan de verhoogde proliferatie, loopt parallel of wordt tegelijkertijd aangestuurd door het tumor suppresor gen *p53* en het myelocytomatosis oncogen *c-Myc*. GATA6 stimuleert ook de differentiatie van de cellen van Paneth en het uitgroeien van precursorcellen tot endocriene cellen, waarschijnlijk door het inhiberen van de transcriptie van het "SAM pointed domain containing ets transcription factor" (*Spdef*) gen en het activeren van de transcriptie van de "delta-like 1" (*Dll1*) en neurogenin 3 (*Ngn3*) genen in de secretoire precursorcellen in het volgroeide ileum. Daarnaast reguleert GATA6 de expressie van specifieke enterocytgenen in de enterocyten van het volgroeide ileum. In tegenstelling tot een afname van het aantal cellen van Paneth in het ileum, resulteert de verwijdering van het *Gata6* gen in de darm tot een toename van het aantal cellen van Paneth en een toename in de transcriptie van genen die coderen voor eiwitten die in de cellen van Paneth tot expressie komen in het jejunum. Waarschijnlijk is dit een compensatoire reactie op het verlies van de cellen van Paneth in het ileum. Verder laten de experimenten in **Hoofstuk 5** zien dat het GATA4 eiwit vele maar niet alle functies van GATA6 in het jejunum kan overnemen. Concluderend laten deze bevindingen zien dat GATA factoren in de dunne darm (1) proliferatie stimuleren, (2) noodzakelijk zijn voor de uitgroei van secretoire precursorcellen tot de juiste gedifferentieerde cellen en (3) de expressie van specifieke enterocytgenen aansturen.

GATA6 stimuleert proliferatie en is verantwoordelijk voor de terminale differentiatie van cellen in het epitheel in het colon

GATA4 komt niet voor in de dikke darm van mens en muis, in tegenstelling tot GATA6 dat wel in de dikke darm van mens en muis aanwezig is. **Hoofdstuk 6** beschrijft, dat GATA6 in alle gedifferentieerde en proliferende cellen in het epitheel van het volgroeide colon van de muis voorkomt. Het GATA6 eiwit stimuleert de proliferatie van het epitheel van de dikke darm. Verder laten de experimenten die beschreven worden in **Hoofdstuk 6** zien dat GATA6 in het colon (1) de expressie van specifieke genen in de enterocyten activeert, (2) de terminale differentiatie van slijmbekercellen stimuleert, terwijl deze naar het oppervlakte epitheel migreren en (3) de dif-

ferentiatie van endocriene cellen naar subtypes reguleert. De wijze waarop GATA6 de processen van gentranscriptie en celdifferentiatie aanstuurt, verschilt tussen het colon (**Hoofdstuk 6**) en het ileum (**Hoofdstuk 5**). GATA6 is noodzakelijk voor de activatie van de transcriptie van het colonocytomarker "carbonic anhydrase 1" (*Car1*) gen in het colon, terwijl GATA6 de transcriptie van het *Car1* gen in het ileum remt. GATA6 is in het colon niet noodzakelijk voor de transcriptie van het familielid van *Car1*, het "carbonic anhydrase 2" (*Car2*) gen, maar remt de transcriptie van *Car2* in het ileum. Verwijdering van het *Gata6* gen in de darm leidt in het colon tot een verlaging van de mRNA en eiwitexpressie van de slijmbekercelmarker MUC2, terwijl dit in het ileum leidt tot een verhoging van de mRNA en eiwitexpressie van MUC2 in de crypten van het ileum. De gen- en ewitexpressie van de endocriene celmarker PYY wordt geïnhibeerd door GATA6 in het colon, terwijl deze wordt geactiveerd door GATA6 in het ileum. Deze bevindingen laten zien dat GATA6 de proliferatie in de dikke darm, zoals in de dunne darm, stimuleert, maar dat GATA6 genexpressie en celdifferentiatie in het colon op een andere manier reguleert dan in het ileum.

RELEVANTIE

Om voedingsstoffen op een efficiënte wijze te kunnen verteren en opnemen, moeten de processen van ontwikkeling, differentiatie, celdistributie en genexpressie van de epitheellaag van de darm op een adequaat gecoördineerde manier worden aangestuurd. Deze processen kunnen helaas eenvoudig verstoord raken door aangeboren darmafwijkingen, darmziektes, darminfecties of darmresecties. Om interventies te ontwikkelen, die een verloren of verminderde darmfunctie kunnen herstellen, is het van essentieel belang om de mechanismen, die ten grondslag liggen aan de ontwikkeling en het in stand houden van een normaal functionerende darm, volledig te begrijpen. Dit proefschrift beschrijft onderzoek dat aantoont dat de evolutionair geconserveerde familie van GATA transcriptiefactoren een belangrijke rol speelt in het aansturen van de proliferatie, differentiatie en expressie van essentiële genen langs de cephalocaudale as in de ontwikkeling en het in stand houden van een normaal functionerend darmepitheel. De transcriptiefactor GATA4, die voorkomt in de proximale dunne darm en niet aanwezig is in de distale dunne darm, reguleert verschillen in absorptie tussen het jejunum en het ileum. Dit bewerkstelligt GATA4 door in de proximale dunne darm de expressie van specifieke enterocytgenen te activeren en de expressie van andere enterocytgenen te inhiberen. De GATA4 en GATA6 eiwitten komen samen voor in de proximale dunne darm, waar ze de proliferatie van het darmepitheel, de differentiatie van de cellen van Paneth en het uitgroeien van secretoire precursorcellen tot endocriene cellen stimuleren. Verder reguleren GATA4 en GATA6 de mate van genexpressie van enterocyteiwitten afhankelijk van waar langs de cephalocaudale as van de darm deze eiwitten zich bevinden. In tegenstelling tot GATA4 komt GATA6 wel voor in de distale dunne darm en het colon. Daar reguleert GATA6 de proliferatie, differentiatie en genexpressie van het darmepitheel. Het toekomstig onderzoek dat wordt aanbevolen in dit proefschrift zal met behulp van nieuwe *in vivo* modellen de mechanismen

verder ontrafelen waarmee GATA4 en GATA6 de processen van proliferatie, differentiatie en genexpressie in het darmepitheel aansturen. De resultaten van dit onderzoek zullen de kennis over de moleculaire processen die ten grondslag liggen aan de ontwikkeling en de instandhouding van een functioneel darmepitheel vergroten en daarmee een noodzakelijk fundament leggen voor de ontwikkeling van interventies, die een verloren of verminderde darmfunctie kunnen herstellen. Verder kunnen deze studies leiden tot nieuwe inzichten in de mechanismen die ten grondslag liggen aan inflammatoire darmziekten, darmkanker en het zelfherstellend vermogen van de darm.

Chapter 9

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In the Grand Lab I was part of the Krasinski Group; a continuously changing group of people that, under the daily supervision of Steve, worked on subprojects of the bigger research plan.

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My dissertation reading committee

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Chapter 10

Curriculum Vitae
Publications and Presentations

CURRICULUM VITAE

Eva Beuling was born in Rotterdam, The Netherlands on October 4th, 1981. She received her primary education at "Het Startblok" in Oude Tonge, and graduated from "De Regionale Scholengemeenschap Goeree-Overflakkee" in Middelharnis in 1999. Because of the *numerus fixus* for medical school in the Netherlands and not getting drawn in the lottery for entering medical school that year, she worked as administrative employee at the department of Radiology at "Het Zuiderziekenhuis" in Rotterdam. The following year, she entered the lottery for medical school again, did not get in, and studied Policy and Management for Health Care at the Erasmus University in Rotterdam as an alternative. In 2001, the Erasmus University in Rotterdam used the decentralized selection instituted by the Dutch government to fill 10% of the *numerus fixus* places and Eva was selected to start medical school that year. As medical student, Eva participated in research projects at the department of Hematology at the Erasmus Medical Center in Rotterdam, testing the potential of the Platelet Function Analyzer (PFA) for determining platelet function in thrombopenic patients; and at the department of Neonatology at the Sophia Children's Hospital in Rotterdam determining the epithelial function in the remnant bowel after surgery for necrotizing enterocolitis in infants. Furthermore, Eva worked as student-assistant at the department of Obstetrics at the Sophia Children's Hospital assisting the nursing staff and continued to work at the department of Radiology at "Het Zuiderziekenhuis". In 2005, Eva attended a research elective at the department of Pediatric Gastroenterology and Nutrition at the Children's Hospital Boston, USA, as graduation project for medical school. She was trained by Stephen D. Krasinski (PhD) and Richard J. Grand (MD) in basic science and worked on a project of Tjalling Bosse (MD, PhD) - at that time, the PhD-student in the laboratory, determining the role of the transcription factor HNF1α in the regulation of intestinal gene expression during development. This research elective led to Eva's first publication which serves as the first chapter of her PhD dissertation. After graduating from medical school at the Erasmus University Rotterdam in November 2005, Eva returned to Boston in 2006 on a Fulbright and VSBfonds scholarship, an international training fellowship from the Nutricia Research Foundation, and with financial support from the Doctor Catharine van Tussenbroek Foundation, the Gerrit Jan Mulder Foundation and the Foundation De Drie Lichten to start her PhD research in the Pediatric Gastroenterology and Nutrition laboratory of doctors Krasinski and Grand. The PhD research Eva conducted in this laboratory is described in the preceding dissertation. While at Children's Hospital Boston, Eva audited the Molecular Biology (MCB 52) and Cell Biology (MCB 54) courses at the Harvard Medical School, Boston. Eva has presented her research results in scientific meetings at the Children's Hospital and the Dana-Farber Cancer Institute in Boston as well as at the gastrointestinal conferences during the Digestive Disease Weeks in Los Angeles (2006), Washington DC (2007), San Diego (2008) and Chicago (2009). The submitted abstract for the gastrointestinal conferences during the Digestive Disease Weeks in Chicago (2009) was presented at a distinguished abstract plenary session, and was rewarded with a travel grant from the WeCare Small Grants program. After her defense, Eva

will start her clinical rotations and pursue her medical career in the hope to combine clinical practice and research in the future.

PUBLICATIONS AND PRESENTATIONS

Publications:

Bosse, T., Fialkovich, J. J., Piaseckyj, C. M., Beuling, E., Broekman, H., Grand, R. J., Montgomery, R. K., Krasinski, S. D., 2007. Gata4 and Hnf1alpha are partially required for the expression of specific intestinal genes during development. *Am J Physiol Gastrointest Liver Physiol*. 292, G1302-14.

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