Intestinal Specific Gene Regulation by Transcription Factors Gata4 and Hnf1α in vivo

Tjalling Bosse

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Intestinal Specific Gene Regulation by Transcription Factors Gata4 and Hnf1α in vivo

Darm specifieke gen regulatie door transcriptie factoren Gata4 en Hnf1α in vivo

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Promotoren:	Prof.dr. H.A. Büller
	Prof. R.J. Grand MD
Overige leden:	Prof.dr. J.B. van Goudoever
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……………..Alleen het kind,
Is nog hetzelfde als voor duizend jaren
Nieuw en verwonderd ligt het rond te staren
Alsof de wereld pas vandaag begint.

O makker in ditzelfde grauw getij
Nog altijd komt het kind tot jouw en mij
Nog altijd kan de wereld nieuw beginnen
In ieder kind kan het opnieuw beginnen,
Zolang God kinderen in ons midden zendt,
Heeft hij zich nog niet van ons afgewend.

Uit het geboorteboekje van mijn moeder

ter nagedachtenis aan mijn broer:
Rutger Bosse

PREFACE

This study was a joint effort between the Division of Pediatric Gastroenterology and Nutrition, Department of Pediatrics, Erasmus MC/Sophia Kinderziekenhuis, Rotterdam, The Netherlands, and the Division of Gastroenterology and Nutrition, Department of Medicine, Children's Hospital Boston, Boston MA, 02115; Department of Pediatrics, Harvard Medical School, USA.

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CHAPTER 1

Introduction
Regulation of intestinal differentiation

Tjalling Bosse[1], Richard J. Grand[2], Hans A. Büller[3] and Stephen D. Krasinski[2]

From the [1]University of Amsterdam, Department of Pediatrics, Amsterdam Medical Center, Amsterdam, 1100DD; The Netherlands; [2]Division of Gastroenterology and Nutrition, Department of Pediatrics, Children's Hospital Boston, Boston MA, 02115; Department of Pediatrics, Harvard Medical School and the [3]Erasmus University Rotterdam, Department of Pediatrics, Erasmus MC/Sophia Children's hospital, Rotterdam, The Netherlands

Introduction

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1 Introduction: Regulation of intestinal differentiation

Significance of studying intestinal differentiation

The mammalian small intestine is responsible for the terminal digestion and absorption of nutrients, water homeostasis, and the elimination of waste products, which in turn, are essential processes for life. These processes however, are easily disrupted by infection, inflammatory processes such as Crohn's disease, cancer, and resection. The small intestine is equipped with specific proteins, such as enzymes to digest nutrients (digestion) and 'transporters' to carry the nutrients into the body (absorption). These tools for digestion and absorption are specifically expressed in the enterocytes of the small intestine and this expression is regulated by a complex of regulatory proteins among which intestinal transcription factors. These regulatory proteins are proposed to be important for intestinal gene expression, differentiation and development and are central to intestinal function. A better insight into the role that specific transcription factors play in these processes will thus complement our understanding of the regulation of intestinal function. Such fundamental knowledge will provide critical insight into disease processes and repair mechanisms of the intestinal epithelium, and identify potential avenues of intervention to correct lost or deficient intestinal function. The research described in this thesis investigates the role of the transcription factors Gata4 and Hnf1α in intestinal gene expression *in vivo*.

1.1 Intestinal development and differentiation

The mammalian small intestine develops from a simple tubular structure in the early embryo to a mature, highly specialized and organized organ system. Beginning on embryonic day (E) 8.5 in mice, anterior and posterior invaginations of the visceral endoderm occur that eventually fuse forming a primitive gut tube lined by an undifferentiated, stratified epithelium. Between E9.5 and E14.5, interactions between visceral endoderm and mesoderm result in organ specification, and the gut tube undergoes considerable longitudinal growth. The primitive gut tube can be divided into three parts: foregut, midgut and hindgut. The mid-gut is destined to become the small intestine, and between E14.5 and E19.5, the midgut endoderm goes through rapid remodeling converting the stratified epithelium into a simple columnar epithelium in a proximal-to-distal wave called cytodifferentiation. During this time, upward growth of the underlining mesenchyme forms nascent finger-like projections (villi) that are separated from one another by a proliferating compartments known as the intervillus epithelium. It is during this important transition that the expression of certain intestine-specific genes is first detected (108). After birth, the mouse epithelium has an immature architecture that goes through considerable morphological and functional changes during the first 3 weeks of life after which it reaches a mature morphology. The timed developmental transitions of the gut are reflected in the well-orchestrated changes in the intestinal gene expression of certain gene sets (also referred to as the

developmental axis). A better understanding in the regulation of these specific intestinal genes will therefore provide insight into the underlying mechanism responsible for intestinal development and differentiation.

1.1.1 Organization of the mouse Crypt-villus structure

The epithelium of the mature intestine is a highly dynamic system in which the cells are continuously renewed by a process involving cell generation, proliferation in the crypts and migration associated differentiation along the villi. A stepwise differentiation process from intestinal stem cells forms the four major cell lineages: absorptive enterocytes, goblet cells, enteroendocrine cells and Paneth cells (Fig. 1).

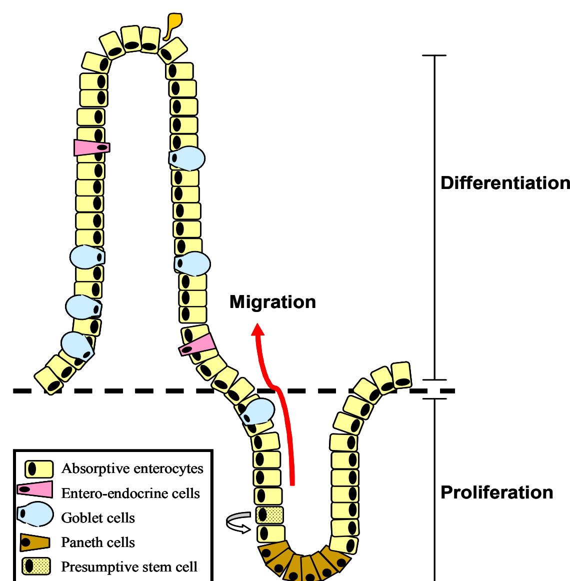

Fig. 1. Schematic representation of intestinal crypt-villus structure. This figure shows a schematic of a " finger-like projection" villus (above the dotted line) and crypt (below dotted line) that characterizes the luminal surface of the small intestine. The four prinicipal cells that originate from the presumptive stem cells are depicted. The epithelium is lined by absorptive enterocytes (light yellow), goblet cells (blue) and enteroendocrine cells (pink) which originate in the crypt and migrate up (red arrow) the villus where they are eventually shed into the lumen. The paneth cells reside in the bottom of the crypts (brown) below the presumptive stem cells. Generally, proliferative immature cells can be found in the crypts, whereas differentiated and mature cells line the villus.

Absorptive enterocytes, comprising approximately 90% of the villus associated epithelium, are polarized cells with an apical microvillus membrane that contains transporters, receptors, and membrane-anchored hydrolases, including lactase-phlorizin hydrolase (LPH) and sucrase isomaltase (SI). Goblet cells synthesize mucins, which provide protection of the epithelium and a medium in which terminal digestion and absorption can occur optimally. Enteroendocrine cells produce a variety of hormones necessary for regulation of intestinal processes, such as peptideYY (PYY), secretin and cholecystokinin (CCK). Paneth cells produce antimicrobial enzymes and peptides such as, cryptidins and defensins (107). While the three other differentiated cell types migrate upward from the upper third of the crypts to the apex of the

villus, the Paneth cells characteristically migrate to the bottom of the crypt where they reside for about 20 days (18, 39). At the tip of the villus, the differentiated epithelial cells become apoptotic and are exfoliated into the intestinal lumen (43). The entire villus epithelium is renewed every 3 days in the mouse (5 days in man) (150). This cell replenishment and migration process along the crypt/villus (vertical) axis guarantees a continuous and rapid renewal of the intestinal epithelium, ensuring maintenance of barrier and absorptive functions, which define the term homoeostasis in this organ.

1.1.2 Regional specialization along the length of the small intestine

For appropriate function it is imperative that the intestine maintain functionally diverse regions along the antero-posterior or horizontal axis. The adult small intestine anatomically starts at the pyloric junction (the most distal point of the stomach) and ends at the ileocecal valve (start of the colon) and is divided into duodenum, jejunum and ileum. Although these transitions are not clearly demarcated, these structures have obvious functional and morphological differences. Many studies, using intestinal isografts, have contributed to the concept that position-appropriate differentiation along the horizontal axis of the small intestine can occur in the absence of exposure to luminal contents, and that such regulation in the small intestine is pre-determined (40). However, little is known about the intrinsic molecular mechanisms that maintain regional diversity in the adult small intestine, which is a principal subject in this thesis (Chapter 4).

The duodenum is the most proximal part of the small intestine and is the first to receive the partly digested chyme from the stomach. Its major function is to provide a medium in which virtually all digestible macromolecules are being digested into forms that are capable of, or nearly capable of, being absorbed by the absorptive enterocytes in the jejunum and ileum. The acidic content of the stomach triggers numerous enteroendocrine cells present in the duodenum to release hormones such as cholecystokinin (CCK) and secretin. These hormones stimulate the secretion of pancreatic fluids and bile into the duodenum. Pancreatic secretions contain digestive enzymes, such as proteases, lipases and amylase, which are responsible for the digestion of proteins, lipids and carbohydrates into small absorbable molecules. Bile acid is the critical component of bile which has the ability to emulsify fat globules to increase lipase action and to solublize fat and fat-soluble vitamins in an aqueous environment by the formation of micelles (aggregates of lipid such as fatty acids, cholesterol and monoglycerides). To protect the epithelium against the acidic fluids, specialized glands (Brunner's glands) secreting basic fluids, are specifically found in the submucosa of the duodenum. Together the epithelial lining of the duodenum is specialized in the digestion of stomach content and the release of hormones that activate gastro-intestinal processes.

In considering the digestive system, the jejunum is central of the three divisions of the small intestine and lies between the duodenum and the ileum. The jejunum is the prime position for the absorption of carbohydrates, proteins and lipids. Therefore the absorptive enterocytes in the jejunum express high levels of integral membrane proteins responsible for the absorption of

disaccharides and peptides. The jejunum differs from the duodenum due to lack of Brunner's glands and a generally lower number of enteroendocrine cells. Together the jejunum functions as the major site of nutrient absorption.

The ileum is the most distal part of the small intestine and ends at the ileocecal valve, a structure that is designed to prevent the backward movement of substances from the large to the small intestine. It is specialized in the absorption of vitamin (B_{12}) and bile acids. To accommodate these functions, the ileal enterocytes specifically express bile acid transporters, and a transporter responsible for vitamin B_{12} absorption. The function of the ileum becomes clinically apparent in patients in whom the ileum is diseased or resected. These patients suffer from disturbed bile-acid metabolism resulting in steatorrhea and eventually gallstone formation. The ileal epithelium contains a relative high number of enteroendocrine cells that produce hormones that repress gastrointestinal processes, and display the highest number of goblet cells. It is currently thought that this relative high number of goblet cells is functionally significant in the protection against the higher bacterial load in the ileum (98). Further, the ileum differs from other regions of the small intestine in its lymphoid tissues. While the length of the intestinal tract contains lymphoid tissue, only the ileum has abundant aggregates of lymphoid cells called Peyer's patches. After passing though the ileum, the luminal content is emptied in the colon in which the terminal phase of digestion and absorption occurs. The functional differences between the three major compartments of the small intestine are well described, and the genes expressed in these regions facilitating the differential functions are well known. The mechanisms, however, responsible for the maintenance of these regional diversities in adult homeostasis are only beginning to be elucidated and are a focus of the present thesis.

1.2 Genetic and epigenetic regulation of gene expression

1.2.1 Transcriptional regulation

When studying the mechanisms of gene regulation processes it is crucial to understand the basics of genetic regulation. In conventional genetic theories the expression of genes in a cell are primarily regulated by the information that lie within the DNA. The DNA sequence can be divided into the genes, which encode for the production of proteins, and the regulatory regions which contain the information for the control of gene expression. In the most simplified model, genes are regulated primarily by a stretch of DNA flanking the genes, called the 5′-flanking region or colloquially the promoter. The anatomy of a typical promoter includes a transcriptional start site, TATA-box, protein binding elements in the proximal promoter and more distal regulatory areas. To initiate the expression of a gene (transcription), a complex of general transcription factors together with RNA polymerase is formed on the TATA box. Specific regulation is accomplished by proteins that recognize and bind the conserved regulatory elements on the proximal promoter of the gene. These proteins, called specific transcription factors, are capable of activating or repressing the transcriptional process. The gene regulation by general-

and specific- transcription factors is further controlled by proteins called co-factors which interact with the transcription factors, but do not bind to the DNA. This mixture of transcription factors and co-factors present in a cell at a certain time, combined with the sequence in the regulatory region of a gene, orchestrates the subset of genes that are expressed. In the central dogma, DNA is transcribed into RNA in the nucleus, the RNA is transported into the cytoplasm where ribosomes translate the RNA into functional proteins. Although the gene expression is regulated at the level of transcription, in some instances genes are also regulated by the steps following transcription. This type of regulation, collectively called "posttranslational regulation", is a mechanism often used for proteins that are required in quick responses to extra-cellular signals.

1.2.2. Epigenetic regulation; histone acetylation and DNA methylation

Next to the genetic regulation by information that lies within the DNA, other forms of regulation by information outside the DNA are starting to be identified and have been collectively called "epigenetic". Epigenetic regulation is the regulation of gene expression that occurs "in addition to" (*epi*-, Greek for "*in addition*") gene sequences in the DNA as described above. Examples of epigenetic regulation are histone acetylation and DNA methylation. The effects of these epigenetic processes on gene regulation are increasingly well understood. Histone acetylation and DNA methylation are further discussed in the following paragraphs.

In the nuclei of eukaryotic cells, genomic DNA is highly folded and compacted by histone and nonhistone proteins in a dynamic polymer called chromatin (122). The distinct levels of chromatin organization are dependent on the dynamic higher order structuring of nucleosomes, which represent the basic repeating unit of chromatin. For each nucleosome, roughly 146 bp of DNA are coiled around an octamer of core histone proteins formed by four histone partners: an H3-H4 tetramer and two H2A-H2B dimers (73). Currently, it is thought that chromatin structure plays an important regulatory role and that multiple signaling pathways converge on histones (149). Modification of the charged histone N-terminus ("tail"), such as acetylation, phosphorylation and methylation, allow regulatable contacts with the underlying DNA. For example, acetylation of lysine residues on the histone tails neutralize the positive charge and thereby decrease their affinity for negatively-charged DNA (50). Consequently, histone acetylation alters nucleosomal conformation (95) which increases the accessibility of transcriptional regulatory proteins to chromatin templates (68) (Fig. 2).

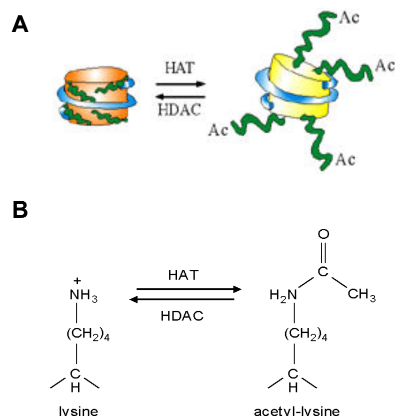

Fig. 2. Histone acetylation (A) Schematic representation of the dynamic state of histone acetylation and deacetylation that is maintained by two enzyme families; histone acetyl-transferases (HATS) and histone deacetylases (HDACs). HAT activity results in acetylated histone tails (Ac) and "opens" the DNA. (B) Acetyl-transferases catalyse the linkage of the acetyl group from acetyl-CoA to the beta-amino group of specific lysine residues, effectively neutralising a positive charge within the N-terminal part of the histone molecule. This modification is reversed by the action of histone deacetylases (HDAC).

When this occurs, complexes of transcriptional factors can bind to the DNA and initiate transcription of the gene. Although this represents the concept of current thinking, little is presently known about the cause and effect relationship between histone acetylation and transcriptional activity or about the underlying molecular mechanism. For example, does transcription factor binding cause acetylation and opening, or does this have to happen first so that transcription factors have access? In chapter 3 of this thesis the acetylation states of the promoters of *LPH* and *SI* in the adult mouse jejunum are investigated.

DNA methylation is also part of epigenetic regulation and involves a type of chemical modification of DNA (addition of a methyl group) without changing the DNA sequence, typically occurring in CpG islands present in the 5'-flanking region (Fig.3).

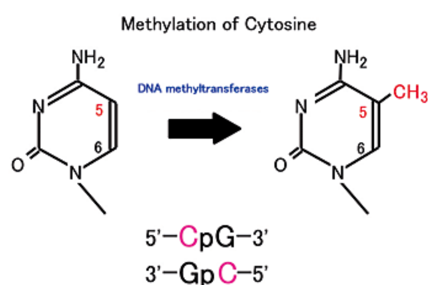

Fig. 3. Mechanism of DNA methylation. 5-Methylcytosine is produced by the action of the DNA methyltransferases, which catalyse the transfer of a methyl group (CH_3) from *S*-adenosylmethionine to the carbon-5 position of cytosine.

A CpG island is a DNA region of at least 200bp with a GC percentage greater than 50%. The methylation state of the CpG island in the regulatory region is closely linked to the chromatin structure of the promoter and subsequently the activity of the gene. Unmethylated regulatory regions are associated with active genes and methylated regulatory regions are associated with inactive genes. It is currently thought that when CpG islands are methylated, specific proteins can bind to the methylated area and can recruit proteins with histone deacetylation activity resulting in closed chromatin and the inhibition of gene expression (156). It has now been shown

that promoter hypermethylation can play a role in cancer formation by inhibiting the expression of tumor suppressor genes (8). Methylation may therefore be a target for future therapies.

1.3 Lactase-phlorizin hydrolase (LPH or lactase) and sucrase isomaltase (SI or sucrase)

LPH and SI are critical intestinal enzymes for nutrient absorption, and are established markers for intestinal differentiation and development; therefore, they serve as model genes in this thesis. LPH and SI are both brush border hydrolases that protrude from the apical membrane of the absorptive enterocyte into the intestinal lumen. LPH is a β-galactosidase responsible for the cleavage of the disaccharide lactose, the principal carbohydrate of mammalian milk, into its absorbable monosaccharides, glucose and galactose. The phlorizin hydrolase activity of LPH also shows specificity for lactosyl residues in glycolipids and phlorizin, a naturally occurring bitter white crystalline glucoside found in the root bark of apple, pear, cherry and mushrooms (20). SI contains isomaltase and sucrase acitivities; cleaves sucrose into glucose and fructose. SI plays a key role in the final degradation of glycogen and starch.

Our interest in the regulation of the lactase gene has emerged from the importance of lactase for human health and the intriguing phenotypic differences observed within certain human populations (explained below). The lactase research however has now evolved into using *LPH* as a model gene to dissect mechanisms responsible for maintenance of intestinal differentiation.

1.3.1 The expression patterns of LPH and SI genes mark the developmental and differentiation phases of the small intestine

The *LPH* and *SI* genes display a complex pattern of intestinal specific gene expression along the vertical, horizontal and developmental axes, indicating that these genes are under tight control mechanisms. *LPH* mRNA expression is first detected in the enterocytes of fetal rats, at E18, coincident with the formation of the first primitive villi (84, 108). Since the occurrence of *LPH* mRNA parallels fetal cytodifferentiation, *LPH* is a marker for this event in the developing gut. At birth and preweaning, *LPH* is highly expressed throughout the immature intestine facilitating the requirement for lactose absorption at this developmental time point. During the third postnatal week in rat, changes occur in the level and distribution of *LPH* and *SI* gene expression. The *LPH* expression per enterocyte is reduced and the expression in the intestine is restricted to the jejunum. In contrast, *SI* gene expression increases from low levels at the time of weaning to high adult levels throughout the small intestine. These enzymatic changes during postnatal developmental are functionally relevant since they coincide with the transition from a milk-based diet, in which the primary carbohydrate is lactose, to a diet of solid foods that contain alpha-disaccharides. Therefore *LPH* and *SI* are excellent markers for the final phase of intestinal

differentiation, since they both are specifically expressed in the small intestine and display highly regulated patterns of expression along the horizontal and temporal axes, indicating common as well as specific control mechanisms (6, 59, 108, 109).

Finally, along the crypt-villus axis (vertical) *LPH* and *SI* are both restricted to the differentiated enterocytes. In fetal rats the mRNA is uniformly expressed on the villi, but not expressed in the intervillus regions. After birth and into adulthood the mRNA is localized to the lower half of the villus, whereas the protein is expressed from crypt-villus junction to the tip of the villi (108). This indicates that the membrane-bound protein is stable for the further lifespan of the enterocyte and illustrates the differentiation-specific nature of *LPH* and *SI* gene expression. Together, these data demonstrate an intricate, highly regulated pattern of expression that coincides with critical mechanisms of gene regulation in the mammalian small intestine. Accordingly, the *LPH* and *SI* genes were used as the principal model for defining the mechanisms of intestine-specific gene expression in this thesis.

1.3.2 Lactase persistence vs. lactase non-persistence

Lactase plays a critical role in the nutrition of human and other mammalian neonates, since this is the only small intestinal brush border enzyme capable of digesting lactose. In the majority of the human population, the pattern of lactase activity during development is characterized by an increase during late fetal period to high levels around birth. In humans, lactase activity decreases around age 5, leading to low activity levels in adulthood. A minority of the human population, however, retains high levels of activity throughout adult life (36, 83). The Northern European population and some nomadic populations of north and central Africa and Arabia (e.g. the Tuareg, Fulbe, Beja and Bedouin people), consider the ability to digest milk in adulthood without complaints as normal. However, in the rest of the world’s population and all mammals, significant milk ingestion results in mild to severe gastrointestinal complaints (abdominal pain, cramps, nausea, flatulence and diarrhea), caused by low lactase levels and the inability to digest lactose (113). This has been misnamed, late onset lactase *deficiency* in the older literature. It is however now clear that low lactase activity is not a deficiency, but a condition found normally in most adult humans. Extensive population studies have indicated that the majority of the world’s populations have low lactase levels in adulthood (36). Therefore the terminology used to describe this phenotype is now named lactase *non-persistence*, and the phenotype of the small group of people in whom lactase levels remain high throughout life is termed lactase *persistence*.

Since all mammals tested display a down-regulation of lactase after suckling, the lactase *non-persistent* phenotype is regarded as the ancestral type (63, 108, 114, 132). The ability for humans to drink milk after suckling only became advantageous after milk from domesticated animals became available. It has therefore been suggested that a mutation event 5000-10,000 years ago (coincident with the domestification of cattle) has resulted in the positive survival benefit for those who could use milk as a nutritional source throughout life (49). In order to be lactase *persistent* it is sufficient to have one active lactase gene (146). By the use of “marker”

single nucleotide polymorphism (SNP) in the coding region of the lactase gene it has been demonstrated that the majority of the lactase mRNA present in lactase *persistent* heterozygous individuals originates from only one allele consistent with their heterozygous status (146). This demonstrates that lactase *non-persistence* is caused by a *cis*-acting transcriptional silencing of the lactase gene and that the individual lactase alleles are regulated independently. The sequence responsible for the silencing of the lactase alleles must therefore be located in the regulatory region of the lactase gene.

Several SNP's have been suggested to play a role in this silencing and have been described in and around the *LPH* gene locus (32, 49). Two particular SNPs have been shown to be tightly linked with lactase *non-persistense* phenotype. A *C* at position -13910 (*C*-13910) upstream of the lactase gene is 100% associated and a *G* at position -22018 (*G*-22018) is more than 95% associated with lactase non-persistence in the Finnish population (31). The *T*-13910 and *A*-22018 variants are associated with lactase persistence. The identification of SNPs associated with the lactase *non-persistence* population may provide the basis for an understanding of the mechanism responsible for this phenotype. Although this phenomenon is not the subject of this thesis, the experiments described complement the knowledge on lactase gene regulation and therefore will aid future studies directed towards an understanding of lactase *non-persistence* in humans.

1.3.3 LPH and SI gene expression is "hard-wired"

Historically there have been three central hypotheses on the mechanism driving *LPH* and *SI* gene expression. These are a) regulation by luminal content, b) regulation by hormonal signals and c) regulation at the level of gene transcription.

The first hypothesis was particularly appealing since the postnatal changes during weaning in *LPH* and *SI* gene expression coincide with dietary changes. However, studies using organ transplants showed that in the absence of luminal content the enzymatic changes still occurred (48, 85). In addition, prolonging the period of suckling in rats caused a delay in decline of *LPH* gene expression, but did not alter the timing of increased *SI* gene expression (67). These studies therefore clearly demonstrate that *LPH* and *SI* gene expression are independent of their substrate.

The second hypothesis (b) was of interest since weaning also coincides with an upsurge in glucocorticoids levels in the growing sucklings (47). In addition, studies in which levels of thyroxine and glucocorticoid hormones were administered in early postnatal rats showed that these hormones cooperatively stimulate *SI* expression and reduce *LPH* expression (154). In contrast, intestinal explant studies showed that the postweaning changes in *LPH* and *SI* activity occurred normally in the transplanted intestine despite constant hormone levels of the adult host (85), indicating an intrinsic program of expression patterns. These and other studies have established that hormonal signals can modulate the expression of *LPH* and *SI*, but the hormonal

effect cannot account for the magnitude of the change that occurs during normal postnatal development.

The remaining hypothesis (c), and the current belief is that *LPH* and *SI* gene expression is regulated at the level of gene transcription which may in part be modulated by hormonal signaling. Evidence for this hypothesis has accumulated throughout the last decade. The first studies in accordance with this hypothesis indicate that the developmental patterns of *LPH* and *SI* in rats (19), and the genetic *LPH* patterns in humans (32) were found to be coordinated with the abundance of its mRNA, findings subsequently extended by us (33, 58) and confirmed by others (34, 45, 53, 63, 72). In addition, we showed that protein activity, protein, mRNA, pre-mRNA, and transcriptional rate for both *LPH*, as well as *SI*, are tightly linked along the length of the intestine and during development as determined by nuclear run-on assays (58). These data demonstrate that LPH and SI biosynthesis is regulated predominantly by transcriptional regulation (58, 109), which has been the basis of the recently published studies.

Transcriptional control of *LPH* and *SI* was further supported by *in vivo* experiments using transgenic mice which showed that both the *LPH* and *SI* promoters direct reporter expression to absorptive enterocytes on small intestinal villi (59, 135, 140). Sequence analysis of the promoter regions of the human, rat, mouse, and pig *LPH* genes (12, 137, 143) as well as the human and mouse *SI* promoter regions reveal consensus binding sites for the transcription factors, Cdx-2, GATA, and HNF-1 (Fig. 4), all of which are expressed in the intestinal epithelium.

Fig. 4. The –100 to –20 bp region of the human (h)(13), rat (r)(143), mouse (m), and pig (p) *LPH* and mouse and human *SI* 5'-flanking sequences. These promoters contain conserved binding sites for GATA (A/TGATAA/G), HNF-1 (GTTAATNATTAAC), and Cdx-2 (TTTAT/C) (*underlined*). TATA-boxes are in *boldface* type. The mouse sequence was obtained from the genetic database of the Celera Discovery System (Rockville, MD) (Dr. M. Fleming, licensee, Dept. Pathology, Children's Hospital, Boston).

Generation of transgenic mice with mutations in the HNF-1 site of a short evolutionarily conserved *SI* promoter (-201) resulted in the attenuation of transgene expression, indicating an important regulatory role for this *cis*-element (15). In a similar approach the GATA site was mutated which led to the identification of a novel putative repressor of *SI* expression Cux/CDP in the distal gut (14). Together these studies demonstrate that information on tissue-, cell-type-, and differentiation-specific *LPH* and *SI* gene expression is "hard-wired" and contained in its 5′-flanking regions. Furthermore, the conserved position of binding sites for GATA zinc finger proteins and the homeobox proteins HNF-1 and Cdx2, in the *LPH* and *SI* promoters, and the close proximity of these sites to the TATA-box and to each other have led to the hypothesis that the factors that bind these sites are important and act in concert to modulate *LPH* and *SI* gene expression *in vivo*. Although Cdx2 is likely an important factor in the regulation of intestinal gene expression, in this thesis we have chosen to specifically study GATA and HNF-1 transcription factors.

1.4 Homeodomain containing transcription factors: Cdx2 and HNF-1 family

1.4.1 Cdx2 transcription factor

Although Cdx2 is not the central focus of the chapters described in this thesis, its importance in differentiation and development of the intestinal epithelium is well established and Cdx2 may function in combination with GATA and HNF-1 factors. *Cdx2* is a homeobox gene and the mouse homologue of the *Drosophila* transcription factor, *Caudal*, which is essential for the formation of posterior structures. In mice, Cdx2 is first detected at E3.5 in extra-embryonic tissue and is later found in the embryo (E8.5) in the posterior gut, tailbud, posterior region of the neural tube, and unsegemented paraxial mesoderm (22). In adult mammals, Cdx2 is highly expressed in small intestine and colon and is also detected in pancreas. Specifically, Cdx2 is expressed in all epithelial cells in crypts and on villi, but only at low levels in Paneth cells at the bases of crypts. Stable transfection of Cdx2 into IEC-6 cells, which are undifferentiated crypt-like cells that do not express Cdx2, results in inhibition of cell growth, induction of marked morphological differentiation, and activation of intestine-specific gene expression. Mice homozygous for the Cdx2 null mutation fail to implant, and die between E3.5 and E5.5, whereas heterozygous knockout mice develop adenocarcinoma of the small intestine and colon (22). Together, these data suggest that Cdx2 is important for implantation during early embryogenesis as well as development and maintenance of a differentiated intestinal epithelium.

Initial studies implicated Cdx2 in enterocyte differentiation due to the regulation of intestinal gene expression. *SI* was the first intestinal gene identified as a Cdx2 transcriptional target, and it remains the best characterized (124, 133). Subsequently, other intestine-specific genes have found to rely upon Cdx2 for their developmental and tissue-specific expression. Cdx2 target genes typically possess one or more copies of a Cdx2-responsive element with a sequence

TTTA(T/C). Other target genes are *LPH* (35, 46, 60, 96, 136, 137), clusterin (123), calbindin-D9K (CaBP9K) (5, 64), carbonic anhydrase 1 (27, 28), HOX C8 (129), the vitamin D receptor (152), guanylyl cyclase C (100), and human apolipoprotein B (69). Cdx2 has also been shown to activate the glucagon promoter in a pancreatic islet cell line (65). Together, these studies indicate that Cdx2 is an essential factor in modulating the expression of many intestinal specific genes.

1.4.2 The HNF-1 transcription factors family

The proteins Hnf1α and -β (hepatocyte nuclear factor-1α and –β) belong to the HNF-1 homeodomain containing family of transcriptional activators that are involved in tissue restricted gene expression of the liver as well as the kidney, intestine, stomach and pancreas (104). Both transcription factors are composed of 3 functional domains: a N-terminal dimerization domain, a POU-homeobox DNA binding domain, and two C-terminal transactivation domains (75)(Fig. 5).

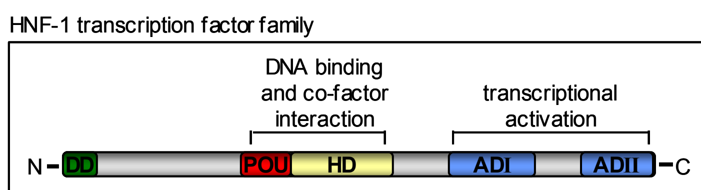

Fig. 5. Schematic representation of the structure of the HNF-1 transcription factor family. HNF-1 proteins contain a dimerization domain (DD), POU-domain (POU), homeodomain (HD), and two activation domains (AD) at the C-terminus.

The DNA binding domain of Hnf1α has the unique feature that it has an insertion of 21 amino acids between the second and third helices of the classical homeodomain. Hnf1α binds as a dimer to the consensus sequence GTTAATNATTAAC (75, 138), although this consensus is highly variable. Dimerizing occurs through the dimerization domain with itself or with Hnf1β, and is stabilized by a cofactor of HNF-1 (DCoH) (44, 76). Hnf1α and Hnf1β share a similar structure with 75% sequence identity in the dimerization domain and 93% in the DNA binding domain. Their C-terminal transactivation domains are more divergent (47%). Both bind the same targets and can act as positive transcriptional regulators; however, Hnf1α is a generally more potent activator. We have also confirmed this in cell culture systems where Hnf1α strongly activated intestinal promoters like *LPH* and *SI*, however Hnf1β did not (further discussed in section 1.6).

Hnf1α is expressed in the polarized epithelia of liver, kidney, intestine, pancreas and stomach in mice (7, 61, 75). The expression pattern of *Hnf1β* overlaps that of *Hnf1α* with a few exceptions. For example, Hnf1β is low in the liver but more abundant in the kidney. In the mouse gut, *Hnf1α* mRNA is uniformly distributed in the stratified epithelium at E10.5 and at E15.5 suggestive for a role in gut morphogenesis (12). In the adult mouse intestine *Hnf1α* mRNA

is expressed in the crypt and lower villus and Hnf1α protein is expressed in the differentiated enterocytes on the villus (14). Preweaning, Hnf1α nuclear protein abundance is low and localized in single cells at the bottom of the villi, suggesting a role for Hnf1α in the developmental transition during weaning (14). Despite these studies, little is known about the exact developmental and horizontal patterns of expression and localization of Hnf1α protein and mRNA. In this thesis (chapter 5) a detailed analysis of Hnf1α expression in mouse development is described.

Hnf1β knockout mice have disorganized visceral endoderm and die at E7.5, and therefore do not provide any information about its role in intestinal differentiation (4, 24). Inactivation of the *Hnf1α* gene in mice has been performed by two laboratories (71, 105). These mice are viable and sterile and displayed decreased growth rate, liver enlargement, renal dysfunction and developed non-insulin-dependent diabetes mellitus. This is consistent with haploinsufficiency of the *Hnf1α* allele in humans, which causes diabetes of the young type-3 (MODY3) (151). The *Hnf1α* knockout mice have therefore been used as a model for this human disease. The *Hnf1α* knockout mice have reduced expression of a variety of liver genes, such as *albumin*, *α1-antitrypsin*, phenylalanine hydroxylase (*pah*), and liver fatty-acid binding protein (*Fabp1*) (1, 105), however *pah* was unaffected in the pancreas where Hnf1α is also expressed suggesting tissue specific regulation (101). Further expression analysis using microchip oligonucleotides revealed alterations in more than 800 liver genes in the *Hnf1α* null mice (117). The enlarged liver of these animals is attributed to the mis-expression of genes in lipid metabolism, and the diabetes is due to alterations in the pathways that regulate beta cell responses to glucose and arginine (105). The overall gastrointestinal morphology is normal in these mice, despite the reduction in intestinal gene expression of *Claudin2* (112) and the apical sodium dependent bile acid transporter (*Asbt*)(117). In this thesis the *Hnf1α* null mice were used to analyze the role of Hnf1α in the expression of intestinal gene expression, specifically *LPH* and *SI*, during intestinal development and differentiation.

1.4.3 Role of Hnf1α in chromatin dynamics

The molecular mechanisms involved in how Hnf1α controls transcription are still poorly understood. However, there is evidence which suggests that Hnf1α may be responsible for regulating the chromatin dynamics of its target genes (101, 106, 111, 121). In *Hnf1α* null mice *pah* expression is attenuated in liver tissue, and this is associated with an abnormal methylation pattern of CpG islands and disappearance of nuclease hypersensitivity sites in the *pah* gene 5′-flanking region in hepatic cells (106). In addition, Hnf1α is required to obtain chromatin opening and transcription of the *α1-antitrypsin* gene in a hepatoma cell line *in vitro* (111). A molecular basis to these findings came from work in which it was found that Hnf1α can interact with proteins with intrinsic histone acetyltransferease (HAT) activity, such as CREB-binding protein p300/CBP-associated factor, and that this HAT activity was relevant for the activation potential of a genome-integrated promoter containing HNF-1 binding sites (121). Finally, using *Hnf-1α*

null mice, Parrizas et. al. (101) demonstrated that promoter occupancy by Hnf1α does not correlate with its requirement for gene expression. They further showed a close linkage between tissue-specific Hnf1α-dependent gene activity and localized acetylation of histone tails. Therefore these studies together suggest that Hnf1α occupies target gene promoters in diverse tissues, but plays an obligate role in transcriptional activation only in cellular- and promoter-specific contexts in which it is required to recruit HAT activity. In this thesis we have utilized chromatin immunoprecipitation assays (ChIP) on mouse jejunal extracts to test the hypothesis that Hnf1α regulates *LPH* and *SI* gene expression by modifying the acetylation state of these gene sets (chapter 3).

1.5 The GATA zinc finger containing transcription factor family

The GATA family is a small group of transcriptional regulators that has been implicated in cell lineage differentiation, organ development, and cell-specific gene expression (62, 78, 82, 90, 99, 103, 119, 148). The GATA family are characterized by an evolutionarily conserved domain consisting of two zinc fingers that direct binding to the nucleotide sequence element (A/T)GATA(A/G) (56, 77). Based on their expression pattern, the GATA family has been divided into two subgroups, Gata1, 2, and 3 and Gata4, 5, and 6. The *Gata1*, *2* and *3* genes are predominantly expressed in the hematopoietic system where they regulate differentiation specific gene expression in megakaryocytes, T-lymphocytes, and erythroid cells (97). Gata4, 5, and 6 have a more diverse pattern of expression and are found in small intestine, heart, liver, lung, and gonads where they play critical roles in regulating tissue-specific gene expression (3, 54, 66, 87, 88, 125). Despite an abundance of knowledge on GATA factors in heart development and hematopoiesis, our understanding of GATA factors in the intestinal epithelium is currently limited. Since Gata4, 5 and 6 are the "intestinal GATAs", they will be the topic of further discussion.

1.5.1 Functional domains of Gata4, 5 and 6

Several functional domains within the GATA family have been identified using protein deletion studies and sequence homology comparisons. The mouse *Gata4*, *5*, and *6* genes all contain 2 activation domains in the C-terminus and two highly conserved zinc finger motifs and an adjacent stretch of basic amino acids, characteristic of the family (Fig. 6).

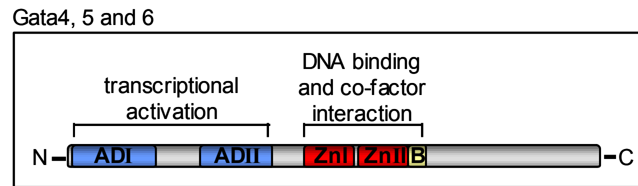

Fig. 6. Schematic representation of the functional domains of Gata4, 5 and 6. Intestinal GATA factors contain a pair of activation domain (AD) in the N-terminus, two zinc fingers (ZnI and ZnII) and a basic region (B) that are required for DNA binding and interactions with other factors.

Within the domains of the zinc-fingers and the adjacent C-terminal basic region, Gata4, 5 and 6 show a high degree of homology (85-95%) (78), suggesting conservation of function and an ancestral relationship. Using mutation and deletion constructs in this region, it has been shown that the C-terminal zinc finger (ZnII) and basic region (B) are sufficient and necessary for DNA binding (89, 142, 153). The N-terminal zinc finger (ZnI) is not required for DNA-binding; it can however can also interact with GATA DNA sequence elements and has been shown to interact with cofactors (89, 148). Within the basic region a strong nuclear localization sequence is present in Gata4 (89). In the N-terminus, two separate transcription activation domains (AD) have been identified by fusing these regions to a yeast Gal4-DNA binding domain and measuring the activity in cell culture (89). Together these studies have mapped several homologous domains with conserved functions within this GATA subfamily, suggesting similar regulation mechanisms.

1.5.2 Differential functions revealed by GATA knockout studies in mice

To reveal the individual functions of these GATA members, mice have been generated in which the *Gata4*, *5* or *6* genes were individually inactivated (57, 62, 79, 82, 90). Mice homozygous for the *Gata4* null allele die by E9.5 because of defects in heart morphogenesis and ventral closure of the foregut (62, 79). Specifically, the ventral closure of the lateral aspects is ineffective in the *Gata4* null mice resulting in a cardia bifida. The malformation of the cardiac structures is likely a secondary effect associated with an intrinsic defect in the folding of the visceral endoderm (92). This interpretation is further supported by the observation that *Gata4* null embryonic stem cells can generate cardiac myocytes but are partially defective in their ability to generate visceral endoderm and definitive endoderm of the foregut (62, 92, 120). Finally, a role for Gata4 in heart development is further suggested by the identification of a deletion in human chromosome 8p23.1 that contains the *Gata4* gene and is associated with congenital heart disease (102). Mice homozygous for a *Gata5* null allele were viable and fertile, but females displayed pronounced genitourinary abnormalities (82), consistent with the observed pattern of *Gata5* expression in the developing genitourinary system. Targeted disruption of the *Gata6* gene in the mouse was lethal very early in embryonic development (E5.5-E7.5), as a result of defects in visceral endoderm

function and subsequent extra embryonic development (57, 90), a phenotype consistent with the expression pattern. Collectively these gene targeting studies indicate that Gata5 is likely not critical in intestinal function. However a role for Gata4 or Gata6 in the intestine cannot be excluded from these studies due to the early lethal phenotypes. To overcome these limitations and investigate the role of these two GATA factors in the intestine, conditional knockout approaches are necessary.

1.5.3 GATA factors in tissue specific gene expression

As stated above, Gata4, 5 and 6 have been implicated as important regulators of gene expression in heart, liver, gonads, lung and gut epithelium. GATA factors regulate the expression of a number of intestinal genes such as *LPH*, *SI*, adenosine deaminase (*ADA*), liver fatty-acid binding protein (*Fabp1*), intestinal fatty-acid binding protein (*Fabp2*), *H+/K+-ATPase*, *trefoil factor* and sodium-hydrogen exchanger isoform 3 (*NHE3*) (2, 14, 30, 35, 38, 55, 94, 128). Moreover, the *C. elegans* GATA, *Elt2*, regulates intestinal specific genes in the gut, extending the role of GATA factors as regulators of intestinal gene expression to include invertebrates (16). Together, these studies indicate that GATA factors are important regulators of essential genes in the small intestine. At the start of this thesis, it was unknown which intestinal GATA was responsible for enterocyte gene expression in mice. However, during the course of this thesis it became clear that Gata4 is the principal GATA factor responsible for enterocyte gene expression in adult mouse intestine.

GATA factors have also been implicated in a variety of other non-intestinal genes. Cardiac genes such as *α-myosin heavy chain*, *cardiac troponin-C*, atrial natriuretic factors (*ANF*), brain natriuretic peptide (*BNP*), *cardiac troponin-I*, cardiac transcription factor *Nkx2.5* and many others (11, 23, 26, 41, 51, 74, 79, 91, 93, 110, 131) have been shown to be regulated by Gata4. The importance for Gata4 in cardiac gene expression is reflected in the phenotype of the *Gata4* null mice (62, 80). Genes expressed in the epithelium of the respiratory tract of the lung such as *surfactant protein A* and thyroid transcription factor-1 (*TTF1*) are regulated by Gata6 (17, 116). That Gata6 is an important lung-specifying factor is further supported by the observation that Gata6 null embryonic stem cells fail to contribute to the lung epithelium in chimeric mouse embryos (90). Liver-specific genes such as *albumin*, vitellogenin II and liver-enriched homeobox *Hex* have also been implicated to be regulated by GATA factors. Furthermore, GATA factors have been implicated in expression of genes in the gonads (118, 134, 144, 147). Collectively, the studies described above suggest that GATA factors are essential regulators of tissue-specific genes expression in a large group of organ systems. However, it is likely that GATA factors regulate tissue-specific gene expression among these different cell types by interactions with other semi-restricted transcription factors or cofactors (see below).

1.5.4 GATA interacting factors

A large cluster of proteins have been described that can interact with GATA factors, including DNA binding factors, co-factors and general transcription factors (9, 10, 21, 25, 29, 70, 81, 86, 115, 134). This wide variety of GATA interaction molecules likely reflects transcriptional mechanisms whereby tissue-specific gene expression is coordinated among various cell types. An example from the cardiac literature is the interaction between Gata4 and Nkx2.5, which is a means to achieve high levels of atrial natriuretic factor (ANF) and cardiac α-actin activity (29, 70, 115). This interaction is mediated by the C-terminal zinc finger of Gata4 and the homeodomain of Nkx2.5. These studies suggest a model in which Gata4 regulates heart-specific gene expression through complexes with other semi-restricted heart-expressed transcription factors. A similar mechanism was found in the regulation of the Mullerian inhibiting substance (MIS) gene promoter in Sertoli cells (134). In these cells the physical interaction of Gata4 with the nuclear receptor SF1 resulted in the synergistic activation of the MIS promoter. We have shown that this paradigm is also applicable to intestinal gene expression as we found that intestinal GATA factors physically associate with Hnf1α resulting in high levels of intestinal specific gene expression (further discussed in section 1.6).

GATA function can also be modulated by interaction with the general transcription machinery (52, 145) and with co-factors (42). A recently identified cofactor of GATA, named friend of GATA (FOG), has been shown to modulate GATA function by interacting with the N-terminal zinc finger. This interaction is conserved in Drosophila where the FOG homologue, *U-shaped*, interacts with *pannier*, a GATA homologue (42). Two FOG co-factors have currently been identified and named Fog1 and Fog2, based on protein structure and expression patterns (127, 130). Fog1 expression corresponds well with the expression of Gata1 (139), while Fog2 generally parallels the expression of Gata4 (130). Targeted disruption of *Fog1* or *Fog2* in mice results in early lethal phenotypes that closely mimic the phenotypes of the *Gata1* and *Gata4* germline knockout mice respectively (103, 126), indicating an essential role for FOG in GATA-dependent gene expression. Interestingly, a targeted mutation in the N-terminal zinc finger of Gata4 specifically disrupts FOG interaction and results in cardiac malformation reminiscent to the germline *Gata4* null mice (25). Together these data demonstrate that FOG is an essential factor in GATA function during heart development. It is therefore possible that FOG co-factors also play a role in GATA function in the intestine. There are, however, currently no reports on FOG expression or function in the small intestine. In chapter 6 we describe the intestinal expression of FOG co-factors and their role in mediating intestinal Gata4 function.

In conclusion, these studies indicate that GATA factors orchestrate the expression of many genes in multiple tissues, and recent evidence suggesting GATA factors as important regulators of gene expression in the intestinal epithelium. The studies discussed above also establish the paradigm that interactions between GATA transcription factors and other semi-restricted transcription factors or co-factors are a means to achieve high levels of tissue-specific gene regulation.

1.5 Complex interactions between tissue restricted transcription factors

The sequence analyses of the proximal promoters of *LPH* and *SI* (Fig. 4) have led to the identification of binding sites for the transcription factor families GATA, HNF-1 and Cdx2 in close proximity to the TATA-box. Indeed, family members of these transcription factors are expressed in the intestinal epithelium (Gata4/5/6, Hnf1α/β and Cdx2) and have been shown to influence the regulation of cellular differentiation in intestinal as well as non-intestinal systems. This resulted in the hypothesis that these transcription factors together are important regulators of *LPH* and *SI* gene expression. Recent efforts by us and others have been directed towards defining the most critical intestinal transcription factors and the mechanism by which they regulate intestinal genes (14, 60, 142). In these studies, we used the conserved proximal promoters of *LPH* and *SI* in cell culture systems to identify the activation potential of GATA, HNF-1 and Cdx2 transcription factors by transient co-transfection assays (60). As a cell culture system, a Caco2 cell line was utilized which is derived from human adenocarcinoma and served as a model for absorptive enterocytes. Caco2 cells differentiate upon confluence exhibiting characteristics of small intestinal absorptive enterocytes, including a microvillus membrane and expression of small intestinal genes, including *LPH* and *SI* (141). As shown in Table 1, the transcriptional responses for short conserved *LPH* and *SI* promoters when co-transfected with individual expression vectors for Gata4, 5 and 6, Hnf1α and β, and Cdx2 show differential effects.

Expression vector	rLPH108	hLPH118	hSI183
pRC-CMV	0.02 ± 0.01^{a}	0.01 ± 0.004^{b}	$0.08 \pm 0.02^{a,b}$
GATA-4	0.28 ± 0.05	0.36 ± 0.06^{a}	0.15 ± 0.02^{a}
GATA-5	0.55 ± 0.11	0.70 ± 0.13	0.40 ± 0.05
GATA-6	0.34 ± 0.06	0.13 ± 0.03	0.12 ± 0.03
HNF-1α	0.34 ± 0.03^{c}	0.32 ± 0.03^{d}	$1.64 \pm 0.25^{c,d}$
HNF-1β	0.07 ± 0.02^{c}	0.02 ± 0.01^{d}	$0.28 \pm 0.01^{c,d}$
Cdx-2	0.08 ± 0.01^{c}	0.14 ± 0.02^{d}	$0.67 \pm 0.13^{c,d}$

Table 1. Transcriptional activities of LPH and SI promoter-reporter constructs co-transfected with expression vectors in Caco-2 cells. Values are mean (± SEM) transcriptional activity (n = 4), expressed as total hGH secreted into the media relative to total CAT in cell lysates per well (mg hGH/Unit CAT activity). The transcriptional activities after co-transfection of individual expression vectors were compared among rLPH108, hLPH118, and hSI183. Values with the same symbol in each row are significantly different; $^{a,b}P<0.05$, $^{c,d}P<0.001$. Courtesy of S.D. Krasinsiki.

Gata5 and Hnf1α had the strongest transactivation potential for the *LPH* promoter, whereas Cdx2 and Hnf1α were the strongest for *SI* activation. The lowest transactivation response was reported for Hnf1β on the *LPH* promoters, however Gata4 was the lowest for the *SI* promoter. These data demonstrated differential effects by multiple transcription factors on the expression of *LPH* and *SI*, indicating a different mechanism of expression.

1.6.1 Evolutionarily conserved mechanism of functional synergy between intestinal GATA factors and Hnf1a.

Among different species and in different organ systems it has been observed that zinc finger families (such as GATA) and homeodomain factors (such as HNF) can physically interact and cooperatively activate target gene expression, suggesting that the interaction between zinc-finger and homeodomains is an evolutionarily conserved mechanism of gene regulation (37, 155). Therefore, it was hypothesized that GATA and HNF-1 proteins in the intestine may cooperatively activate intestinal genes. To test this hypothesis Gata5 and Hnf1α were co-transfected singly and together with a human *LPH* promoter-reporter vector in HeLa cells. Gata5 and Hnf1α were used, because they had the strongest transactivation effect on both the *LPH* and *SI* promoters when co-transfected individually as described above. Indeed, when Gata5 and Hnf1α were cotransfected together they activated the *LPH* promoter more strongly than the additive effect of each of the expression vectors co-transfected singly as shown in Figure 7.

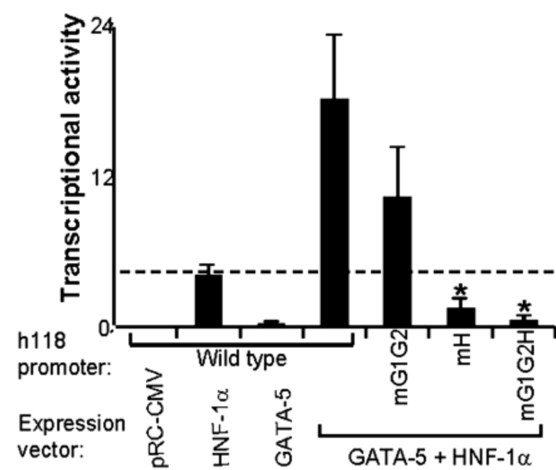

Fig. 7. GATA/Hnf1α synergistic activation of the human *LPH* promoter requires intact HNF-1-binding sites on the DNA. Transient cotransfection assays were carried out in HeLa cells using wild-type Gata5 and Hnf1α expression vectors cotransfected with wild-type and mutant promoter-reporter constructs. The h118mG1G2 promoter-reporter construct contains mutations in both GATA sites, whereas h118mH contains a mutation in the HNF-1-binding site. The h118mG1G2H construct contains mutations in all three binding sites. The *dashed line* indicates the sum of transcriptional activities of Gata5 and Hnf1α expression vectors cotransfected individually with h118wt. Transcriptional activities that extend above the *dashed line* indicate synergistic activation. Data are means ± S.E. ($n = 5$). *, $p < 0.05$ compared with h118wt cotransfected with Gata5 and Hnf1α. Courtesy of H.M. van Wering.

In addition figure 7 shows that this cooperative activation required an intact HNF-1 site; however, an intact GATA site was not necessary. The same cooperative response on the *LPH* promoter was found in chapter 2 of this thesis where Gata4 was used, indicating that both Gata4 and Gata5 are equally capable of cooperatively activating the *LPH* promoter in combination with Hnf1α. This stronger activation by the combination of GATA and Hnf1α proteins can be defined as *functional synergy*. Therefore it can be concluded that GATA factors together with Hnf1α synergistically activate the *LPH* promoter.

Next, a thorough characterization of the molecular mechanism of the functional synergy between GATA factors and Hnf1α factors was undertaken using Gata5 as a model (142). These studies revealed that Gata5 and Hnf1α physically associated both *in vivo* and *in vitro* and this

interaction was required for the Gata5/Hnf1α cooperative activation. Additionally, this physical interaction was mediated by the C-terminal zinc finger and basic region of Gata5 and the homeodomain of Hnf1α. Deletion of the Hnf1α activation domains resulted in an absolute loss of cooperativity, whereas a deletion of Gata5 activation domains resulted in a reduction, but not an attenuation, of cooperativity. In this thesis, an identical cooperativity and physical interaction with Hnf1α was found for Gata4 (Chapter 2).

1.6.2 Intestinal specific expression patterns of Gata4, Hnf1a and Cdx2

In order for transcription factors to cooperatively activate intestinal genes, their expression pattern must overlap with each other and with the expression of the intestinal genes they activate. Using this argument, Boudreau et al. (14) compared the postnatal expression patterns of Gata4, Hnf1α and Cdx2 with the expression of SI in the mouse intestine. Gata4, Hnf1α and Cdx2 were chosen because they were the primary factors from adult mouse intestinal epithelium that interacted with the *SI* promoter, providing key data delineating the intestinal factors critical for *SI* gene expression. Immunolocalization studies revealed that Hnf1α protein is rarely detected in suckling mice and becomes progressively expressed in the villous epithelial cells during the suckling/weaning transition, a pattern correlating with the postnatal induction of *SI* gene expression. Gata4 protein was detected in differentiated epithelial cells on villi of the proximal intestine; however, lower levels were noted in the distal ileum. This low level of Gata4 expression in the distal ileum was further defined in this thesis (chapter 2 and 4) and correlates well with the absence of *LPH* gene expression in this segment. Cdx2 proteins are detected on the entire villi and in colon at all ages, paralleling the horizontal *SI* expression pattern in adults. In this report it is suggested that the most important transcription factors for the temporal and position-dependent regulation of *SI* in the mouse intestine are Gata4, Hnf1α and Cdx2 (14). While, this study provided the first intestinal protein expression data on these transcription factors, the presented data are limited. A detailed analysis of the intestinal expression of Gata4 and Hnf1α on both mRNA and protein level during intestinal differentiation and development will be necessary to better correlate these factors with the differentiation processes of the small intestine, and these data are provided in this thesis (chapters 2,4 and 5).

1.7 Specific Aims of thesis

This introduction describes how during intestinal development and in the adult tissue the cellular phenotypes are defined by the expression of specific genes in the individual cells. The sets of genes expressed in the intestinal epithelial cell are principally determined by transcriptional initiation and shift in well-orchestrated patterns during development, differentiation, and adaptive processes in the intestinal mucosa. Understanding the molecular mechanisms that regulate transcription of the cellular gene sets is the foundation for understanding intestinal development and differentiation events. Current understanding of transcriptional regulatory machinery in the small intestine is based on the analysis of a few genes expressed in restricted cellular and developmental patterns. These genes encoding the disaccharidases SI and LPH have many attributes that makes them excellent marker genes for the investigation of the mechanisms that direct intestine-specific gene expression. Their expression is limited to the cells of the small intestine enterocytic lineage. In addition, they have a complex pattern of expression that is intrinsically directed by information in the 5′-flanking region. The discovery of conserved binding sites for GATA, HNF-1 and Cdx2 transcription factors in the proximal promoters of *LPH* and *SI* has directed our research towards these transcription factor families. The overlapping expression of these transcription factors in the small intestine has led to the paradigm that interactions between these factors may be a means to achieve high levels of intestine specific gene expression. Recently we demonstrated that intestinal GATA factors and Hnf1α physically associate and functionally cooperate in the activation of the *LPH* promoter *in vitro*. During the course of this thesis, Gata4 emerged as the critical absorptive enterocyte GATA factor. While *in vitro* approaches are necessary to understand underlying mechanisms of gene expression, subtractive technology is the preferred approach to define the function of a transcription factor *in vivo*. Therefore, the central aim of this thesis was to translate the *in vitro* findings of cooperativity between Gata4 and Hnf1α into an *in vivo* model using knockout mouse technology for both Gata4 and Hnf1α.

Specific Aim 1:

To determine the mechanism that establishes independent functions for individual GATA factors in the activation of the *LPH* promoter

Specific Aim 2:

To study the importance of Hnf1α and its binding site for the regulation of *LPH* and *SI* gene expression *in vivo*, and delineate the function of Hnf1α-dependent recruitment of histone acetylase activity.

Specific Aim 3:
To define the role of Hnf1α in intestinal gene expression using a germline *Hnf1α*$^{-/-}$ mouse line as an experimental model.

Specific Aim 4:
To establish a conditional *Gata4* knockout mouse as a model to study the role of Gata4 in the maintenance of intestinal differentiation.

Specific Aim 5:
To delineate the importance of FOG co-factors for mediating Gata4 function in the intestine by defining the regulatory pathways altered *in vivo* by a *Gata4* knock-in that cannot bind FOG co-factors.

Specific Aim 6:
To define the role of Gata4 and Hnf1α during intestinal differentiation and development using the differentiation markers *LPH* and *SI*.

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CHAPTER 2

Complex regulation of the lactase-phlorizin hydrolase promoter by Gata4

Herbert M. van Wering[1], Tjalling Bosse[2], Anna Musters[2], Evelien de Jong[1], Naomi de Jong[2], Caroline Hogen Esch[3], Francois Boudreau[4], Gary P. Swain[5], Lauren N. Dowling[6], Robert K. Montgomery[6,7], Richard J. Grand[6,7], Stephen D. Krasinski[6,7,8]

From [1]the Department of Pediatrics, Free University of Amsterdam, Amsterdam, The Netherlands 1081HV, [2]the Department of Pediatrics, University of Amsterdam, Amsterdam, The Netherlands 1100DD, [3] the Department of Pediatrics, Leiden University, Leiden, The Netherlands 2300RC, [4]the Departement d'Anatomie et Biologie Cellulaire, Faculte de Medecine, Universite de Sherbrooke, Quebec J1H 5N4, Canada, [5]the Division of Gastroenterology, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, 19104,the [6]Division of Gastroenterology and Nutrition, Department of Medicine, Childrens Hospital, Boston, Massachusetts, 02115, [7]the Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, 02115 and [8]the Dorothy R. Friedman School of Nutrition Science and Policy, Tufts University, Boston, Massachusetts, 02111.

ABSTRACT

Lactase-phlorizin hydrolase (LPH), a marker of intestinal differentiation, is expressed in absorptive enterocytes on small intestinal villi in a tightly regulated pattern along the proximal-distal axis. The *LPH* promoter contains binding sites that mediate activation by members of the Gata4, -5, -6 subfamily, but little is known about their individual contribution to *LPH* regulation *in vivo*. Here, we show that Gata4 is the principal GATA factor from adult mouse intestinal epithelial cells that binds to the mouse *LPH* promoter, and its expression is highly correlated with that of *LPH* mRNA in jejunum and ileum. Gata4 cooperates with Hnf1α to synergistically activate the *LPH* promoter by a mechanism identical to that previously characterized for Gata5/Hnf1α, requiring physical association between Gata4 and Hnf1α, and intact HNF-1 binding sites on the *LPH* promoter. Gata4 also activates the *LPH* promoter independently of Hnf1α, in contrast to Gata5, which is unable to activate the *LPH* promoter in the absence of Hnf1α. Gata4-specific activation requires intact GATA binding sites on the *LPH* promoter, and was mapped by domain swapping experiments to the zinc finger and basic regions. However, the difference in the capacity of Gata4 and Gata5 to activate the *LPH* promoter was not due to a difference in affinity for binding to the GATA binding sites on the *LPH* promoter. These data indicate that Gata4 is a key regulator of *LPH* gene expression that may function through an evolutionarily conserved mechanism involving cooperativity with Hnf1α and/or via a GATA-specific pathway that is independent of Hnf1α.

INTRODUCTION

The absorptive enterocyte, comprising over 95% of the cells on the villus epithelium, is a highly differentiated columnar cell that expresses specialized proteins required for intestinal function, including digestive enzymes, membrane transporters, and a variety of receptors and carriers (18,68). These proteins also display complex topographic patterns resulting in distinct functions along the length of the small intestine. Lactase-phlorizin hydrolase (LPH), the enzyme responsible for the digestion of milk lactose into the absorbable monosaccharides, glucose and galactose, is an excellent model for the study of intestine-specific gene expression and intestinal differentiation because in adult mammals LPH is expressed only in the small intestine (45) and is confined to absorptive enterocytes on villi, not proliferating cells of the crypt compartment (57,58). *LPH* demonstrates 'positional' regulation, as exhibited by a tightly controlled pattern of expression along the proximal-distal axis of rodents (6,16,25,27,35,58,69) and humans (52), with high levels in the jejunum and proximal ileum, and reduced levels in the duodenum and distal ileum. The pattern of *LPH* gene expression is regulated mainly by gene transcription (25). In transgenic mice, 1 kb of the pig *LPH* 5′-flanking sequence (69) and 2 kb of rat *LPH* 5′-flanking sequence (27,35) are sufficient for tissue-, cell-type-, and differentiation-specific transgene expression. The *LPH* 5′-flanking sequence of mice, rats, and humans contains conserved GATA, HNF-1, and Cdx binding sites within 100 bp of the transcription initiation site (Fig. 1) suggesting that the factors that bind these sites may be important and act in concert. Recent studies have focused on the role of the Gata4, -5, -6 subfamily of transcription factors in the regulation of *LPH* gene expression (13,15,28,73).

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          -100                                                           -40           -25
            •    GATA       HNF-1           GATA         Cdx              •         Cdx •
Human     5'-ATCATAGATAACCCAGTTAAATATTAAGTCTTAATTATCACTTAGTATTTTACAACCTCAGTTGCAGTTATAAAGT-3'
                                               3'-AATAGT-5'  3'-AAATG-5'
Rat       5'-TATCTATCCTAGATAACCCAGTTAAATATTGTGTGGATAATCACTATGTTTTACAGCCTTGGCTGTGCTCATAAAG-3'
                                                             3'-AAATG-5'
Mouse     5'-TATCTGTCCTAGATAACCCAGTTAAATATTGTATGGATAATCACTGTATTTTACAGCCTTGGCTGTGCTCATAAAG-3'
                                                             3'-AAATG-5'
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Fig. 1: The *LPH* gene contains conserved transcription factor binding sites in the 5′-flanking sequence. The -100 to -25 bp regions of the human (2), rat (75), and mouse (Celera mouse genomic DNA database, courtesy of Dr. M. Fleming, Department of Pathology, Children's Hospital Boston) *LPH* 5′-flanking sequence are shown. GATA, HNF-1 and Cdx binding sites are *underlined* and the TATA-box is in *bold-face type*. Previous studies have shown that there are two functional GATA sites in the human *LPH* promoter, but only one in the rat and mouse *LPH* promoters (28).

The Gata4, -5, -6 subfamily of GATA factors, like the Gata1, -2, -3 subfamily, contains a pair of zinc fingers of the four-cysteine type and an adjacent basic region that together mediate binding to the consensus DNA sequence, WGATAR. Gata1, -2, and -3 are expressed in developing blood cells and are critical for hematopoiesis (54), whereas Gata4, -5, and -6 are

expressed in more diverse patterns that include cardiac tissue, small intestine, stomach, liver, lung, spleen, ovary, testis, and bladder (1,21,32,47,48,66) where they are thought to regulate tissue-specific gene expression (42). Gene knockout studies indicate that Gata4 and Gata6 are critical for embryonic development as *Gata4* null mice die during early cardiogenesis (31,43) and *Gata6* null mice fail at the time of gastrulation (24,51). *Gata5* knockout mice display minor genitourinary abnormalities in females, but normal intestinal structure (44); intestinal gene expression has not been reported. In post-weaning and adult mice, *Gata4*, *-5*, *-6* mRNAs are expressed throughout the small intestine (1,12), although only Gata4 and Gata6 protein have thus far been detected (5,10,12). Gata4, -5, -6 subfamily members have been shown to bind and activate the promoters of several intestinal genes including *LPH* (13,15,28,73), sucrase-isomaltase (*SI*) (4,28), intestinal fatty acid binding protein (*Fabp2*) (17), liver fatty acid binding protein (*Fabp1*) (9), sodium-hydrogen exchanger isoform 3 (*Nhe3*) (22), adenosine deaminase (*Ada*) (12) and trehalase (*Treh*) (53). GATA factors may interact with hepatocyte nuclear factor-1α (Hnf1α), a homeodomain protein, to synergistically activate intestinal gene promoters (4,10,28,73). We have found that Gata5 physically associates with Hnf1α to synergistically activate the *LPH* promoter by an evolutionarily conserved mechanism (73), but is incapable of activating the *LPH* promoter independently of Hnf1α (28). Together, these studies support an important role for GATA factors in the regulation of intestinal gene expression.

The goal of the present study was to define the importance and underlying mechanism of action of Gata4 in the regulation of *LPH* gene expression. Our data show that Gata4 is the principal intestinal GATA factor that binds to the GATA binding site on the mouse *LPH* promoter, and its expression is highly correlated with that of *LPH* in the jejunum and ileum. Gata4 activates the *LPH* promoter synergistically with Hnf1α, similar to Gata5, as well as independently of Hnf1α, distinguishing it from Gata5. Structure-function studies reveal that the zinc finger and basic regions mediate differential functions between Gata4 and Gata5, but the underlying mechanism is not due to differences in affinity for binding to the GATA sites on the *LPH* promoter. These data indicate that Gata4 is a key regulator of *LPH* gene expression, and reveal for the first time independent functions among individual intestinal GATA factors.

MATERIALS AND METHODS

Plasmids. Previously characterized expression vectors for mouse Gata4 (31), Gata5 (48) (gifts of M. Parmacek, University of Pennsylvania) and Hnf1α (30) (gift of G. Crabtree, Stanford University) were obtained for these studies. Since the original Hnf1α expression vector replicates inefficiently during bacterial amplification, the Hnf1α coding region was subcloned into pcDNA1, as previously described (73).

For transfection studies, the human *LPH* promoter containing 118 bp of 5′-flanking region was fused 5′ to the human growth hormone reporter (h118wt) (28). This promoter region

contains two GATA binding sites, an HNF-1 site, and a Cdx site, as previously described (28,74) (Fig. 1). In specified experiments, previously constructed plasmids (28) were used in which mutations were introduced into the two GATA sites (h118mG_1G_2), the HNF-1 site (h118mH), or in all three sites together (h118mG_1HG_2). Mutations introduced into these sites have been previously shown to disrupt specific protein-DNA interactions (28). pRC-CMV (Invitrogen, Carlsbad, CA) served as a negative control expression vector for all co-transfection experiments.

To construct plasmid templates for the synthesis of RNA probes for RNase protection assays, mouse cDNA sequence (*LPH*, bp 285 to 535; *Gata4*, bp 456 to 736; *Hnf1α*, bp 2936 to 3174) was amplified and subcloned into pBluescript II KS(+). Templates for antisense probes were linearized and transcribed using [α-^{32}P] UTP (800Ci/mmol, PerkinElmer Life Sciences, Boston, MA) - *LPH* antisense: *Xba*I, SP6 polymerase; Gata4 antisense: *EcoR*I, T3 polymerase; Hnf1α antisense: *Kpn*I, T7 polymerase. A mouse β-actin probe (27) was used as a control for tissue RNA.

All plasmids used in these studies were amplified in *E. coli* DH5α under ampicillin selection and isolated under endotoxin-free conditions using the High Purity Plasmid Maxiprep System (Marligen Biosciences, Inc, Ijamsville, MD).

Mice. Approval was obtained from the Institutional Animal Care and Use Committee for all experiments involving mice. Mice were housed under standard conditions in the Animal Research at Children's Hospital (ARCH) animal facility and provided food and water *ad libitum*. All study animals were 8-16 weeks of age, and all tissue was collected between 1300 and 1600 hr to avoid any fluctuation in gene expression due to circadium cycles (56).

Isolation of nuclear extracts from intestinal epithelium. Nuclear extracts were isolated from the intestinal epithelium of adult mouse intestine as previously described (55). Mice were anesthetized with avertin anesthesia (240 mg/kg), the abdomens opened longitudinally, and the intestine was removed and transferred to a glass plate on a bed of ice. The samples used for experimentation included 8 cm segments centered at the geometric center of the small intestine (jejunum), or pooled samples from the first cm adjacent to the pylorus (segment 1), two cm centered at the first and second quarter junction (segment 2), two cm centered at the geometric center (segment 3), two cm centered at the third and fourth quarter junction (segment 4), and 3 cm adjacent to the ileocecal junction (segment 5). Segments were isolated, rinsed with ice-cold PBS containing fresh 2 mM phenylmethanesulonyl fluoride (PMSF), 1 mM benzamidine, and 60 IU/µl aprotinin, opened longitudinally, and cut into 5-mm cross-sectional segments. The segments were transferred to a 50 ml tube containing 5 ml of BD Cell Recovery Solution (BD Bioscience, Bedford, MA), 2 mM PMSF, 2 mM benzamidine and 120 IU/µl aprotinin, and incubated at 4°C for 18-20 h. After gentle manual inversion for 1 min, the mucosal pieces were removed and a sample was visualized under trypan blue staining to confirm the dissociation of epithelial cells while the remaining cells were collected by centrifugation and washed with ice-cold PBS. Nuclear extracts were then isolated as previously described (65), and the quality was

determined by expected GATA and HNF-1 complex formation in EMSAs and by detection of Gata4 and Hnf1α using Western analysis.

In vitro transcription and translation. Unlabeled and labeled wild type and mutated Gata4, Gata5, and Hnf1α proteins were synthesized as previously described (73) using the TNT™ transciption/translation system (Promega, Madison, WI). Labeled proteins were synthesized using [^{35}S]-methionine (Redivue, Amersham-Pharmacia Biotech, Piscataway, NJ).

Electrophoretic mobility shift assays (EMSAs). To define protein-DNA interactions, EMSAs were carried out as previously described (28) using oligonucleotides shown in Fig. 2A as probes and/or competitors. For competition or supershift EMSAs, competitors or antibodies (0.1 μg/μl), respectively, were pre-incubated with the nuclear extract or TNT™ protein for 20 minutes prior to the addition of the probe. The antibodies used in EMSAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) as the concentrated gel shift stock (Gata4, sc-1237X; Gata5, sc-7280X; Gata6, sc-7244X; Hnf1α, sc-6547X). In certain experiments, band densities from autoradiography were quantified using the Chemi-Doc gel document system and Quantity One software (Bio-Rad, Hercules, CA). All experiments were conducted on at least three different animals.

RNase protection assays. The quantitative pattern of *LPH*, *Gata4*, and *Hnf1α* mRNA expression was defined by RNase protection assays using a previously described protocol (25,27). For proximal-distal analysis, five 1-cm segments were taken from regions as described for the isolation of nuclear extracts (the only distinction being that segment 5 was the most distal cm adjacent to the ileocecal junction). Total RNA was isolated using the RNeasy™ kit (Qiagen, Valencia, CA), quantified by optical density at A260 nm, and checked for degradation on an agarose gel. [^{32}P]-labeled probes were hybridized at 68°C in 50% formamide overnight, digested with RNase A and T1, and the protected fragments were separated on 6% denaturing polyacrylamide gels and revealed by autoradiography. All experiments were conducted on at least three different animals.

Western analysis. To quantify Gata4 and Hnf1α protein along the proximal-distal axis, western analysis was conducted. Nuclear extracts of mouse intestine (15 μg) were electrophoresed in 10% SDS-polyacrylamide gels, and transferred onto Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA) by electroblotting. After transfer, gels were stained with Coomassie Blue to check for transfer efficiency. The membranes were blocked at room temperature for 1 hour or overnight at 4°C in 5% nonfat dried milk in PBS. The antibodies used in Western analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and included goat anti-Gata4 (sc-1237, 1:2000 dilution) and goat anti-Hnf1α (sc-6547, 1:3000 dilution). Gata4 and Hnf1α were visualized with horseradish peroxidase-linked secondary antibodies and the Supersignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL) following the manufacturers instructions. To verify equal loading of samples, the blots were stripped in 62.5 mM Tris-HCl (pH 6.7), 2% SDS and 100 mM β-mercaptoethanol at room temperature for one hour, blocked, and reprobed with mouse

monoclonal anti-β-actin (Sigma-Aldrich, St. Louis, MO) at 1:3000. Experiments with non-immune serum were used as negative controls. Positive controls were assays conducted using TNT™ proteins. All experiments were conducted on at least three different animals.

Immunohistochemistry. To define co-expression of Gata4 and Hnf1α, and to verify their proximal-distal expression patterns, immunohistochemistry was performed on serial sections of adult mouse jejunum (segment 2) and ileum (segment 5). Intestinal segments were isolated, rinsed in ice-cold PBS, fixed in 4% paraformaldehyde in PBS, embedded in paraffin as longitudinal segments, and sectioned (6 μm) onto Superfrost Plus™ (Fischer) slides. After heating for 30 min at 37°C and deparaffinization, sections were boiled in a microwave for 6 min in 10 mM sodium citrate (pH 6.0), rinsed, blocked with protein blocking agent (Coulter-Immunotech, Miami, FL) for Gata4 immunostaining, or TENG-T (10 mM Tris, pH 7.5, 5 mM EDTA, 0.15 mM sodium chloride, 0.25% gelatin, 0.05% Tween-20) for Hnf1α immunostaining, and incubated overnight at 4°C with affinity-purified primary antibodies from Santa Cruz Biotechnology. Goat anti-Gata4 (sc-1237) and goat anti-Hnf1α (sc-6547) were each used at a concentration of 0.2 μg/ml. The primary antibodies were visualized using a biotinylated secondary antibody and an avidin-horseradish peroxidase conjugate (Vectastain ABC kit, Vector Labs). Signal development was achieved using 3,3´-diamino benzidine (Sigma) for 4-6 min. The tissue was lightly counterstained with methyl green. Sections were imaged under bright field microscopy using SPOT™ image capture software (Diagnostic Instruments, Inc, Sterling Heights, MI). All experiments were conducted on at least three different animals.

Cell culture and transient co-transfection assays. To characterize the function of GATA factors on the *LPH* promoter, transient co-transfection assays were conducted. HeLa cells were used because these cells do not synthesize endogenous Hnf1α (28), and are therefore a model for both Hnf1α-independent and Hnf1α-dependent (co-transfected) activation. Cells were transfected using Effectene™ reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Optimal conditions were 1 μg total DNA (0.4 μg of promoter-reporter construct, 0.2 μg of expression vector when included, and pBluescript as carrier) and 4 μl of Effectene™ reagent for transfection in 6-well plates. Media were replaced after 24 h, and collected for human growth hormone analysis after an additional 24 h. Human growth hormone was quantified using an [^{125}I] radioimmunoassay kit (Allegro hGH, Nichols Institute Diagnostics, San Clemente, CA). All plates were confluent at the time of harvest. To control for transfection efficiency, all transcriptional activities were expressed relative to pXGH5, as documented previously (73). All HeLa cells used in the present experiment were mycoplasma-free, as determined using the Mycoplasma Detection Kit (ATCC, Manassas, VA).

Site-directed mutagenesis. To delete or disrupt specific structures within Gata4, site-directed mutagenesis as originally described by Kunkel (29) and modified (73) was carried out on the Gata4 and Gata5 expression vectors. Mutagenic oligonucleotides are outlined in Fig. 2B as the forward strand for clarity, although the reverse strand was used for all uracil templates. G4(-AD) resulted in the insertion of a *BamH*I site and a new start codon. The activation domains,

which are flanked by *BamH*I sites after mutagenesis, were removed by a *BamH*I digest followed by an intramolecular ligation. G4(C290S) resulted in a cysteine-to-serine substitution in the C-terminal zinc finger at amino acid position 290. G4(AD/Nhe), G4(CTD/Nhe), G5(AD/Nhe), and G5(CTD/Nhe) incorporated NheI sites between the activation domains and zinc fingers, or the zinc fingers and C-terminal domains of both Gata4 and Gata5 for domain swapping experiments. The zinc finger and basic regions were removed by digestion with NheI, interchanged, re-ligated and checked for proper orientation and codon integrity by sequencing.

GST pull-down assays. To partially map the structures necessary for protein-protein interactions, GST pull-down assays were carried out as previously described (4,73). GST-Hnf1α protein synthesis was induced in *E. coli* DH5α using 0.1 μM isopropyl-1-thio-D-galatcopyranoside for 3 h (62), and purified using GSH-Sepharose beads (Amersham) according to the manufacturer's instructions. For pull-down assays, GST-Hnf1α was incubated with labeled wild-type or mutated Gata4 and glutathione-coated beads in binding buffer (73) for 2 h at 4°C. The beads were washed 5 times in PBS, and bound proteins were released by boiling in SDS sample buffer and resolved by SDS-PAGE.

A

Oligonucleotides for EMSA

Name	Oligonucleotide	Description
XGATA	5'-GGAGATCCCTGTAC**AGATAT**GGGGAGAC-3'	GATA binding site from *Xenopus* I-FABP gene
hG_1	5'-GTGATCAT**AGATAA**CCCAGTTAAA-3'	5' GATA binding site in human LPH promoter
hG_2	5'-ATACTAAG**TGATAA**TTAAGACTTA-3'	3' GATA binding site in human LPH promoter
mG_1	5'-TCTGTCCT**AGATAA**CCCAGTTAAA-3'	GATA binding site in mouse LPH promoter

B

Mutagenic Oligonucleotides

Name	Oligonucleotide	Mutation	Effect
G4(-AD)	5'-GAGCATCCTGG*GGATCC***ATG**GCTGCCGCCTC-3'	*BamHI*, **Start**	Deletion of activation domains
G4(C290S)	5'-GGTGAGCCTGTA**TCT**AATGCCTGCGGC-3'	**C290S**	Cysteine-to-serine substitution
G4(AD/Nhe)	5'-CGGCGACGG*CGATCG*CGGCCGGGGAA-3'	*NheI*	Incorporation of NheI site between activation domains and zinc fingers
G4(CTD/Nhe)	5'-ACCACTCTGGGAG*CGATCG*AGGTCACCACG-3'	*NheI*	Incorporation of NheI site between zinc fingers and C-terminal domain
G5(AD/Nhe)	5'-CGGGGAAGCACC*CGATCG*CCTCGGAAGCT-3'	*NheI*	Incorporation of NheI site between activation domains and zinc fingers
G5(CTD/Nhe)	5'-GTTTGTGGTGT*CGATCG*AGAGGGTGAGAGGG-3'	*NheI*	Incorporation of NheI site between zinc fingers and C-terminal domain

Fig. 2. Oligonucleotides used in this study. A. EMSA oligonucleotides. The forward strand is shown. GATA binding sites are indicated in *boldface* type. hG_1 corresponds to the 5′ GATA site and hG_2 depicts the 3′ GATA site in the human *LPH* gene; mG_1, the only functional GATA binding site in the proximal mouse promoter, is conserved in position with that of hG_1. B. Mutagenic oligonucleotides. The forward strand sequence is shown. Incorporation of new codons is indicated in *boldface* type and new restriction sites are shown in *underlined italics*.

Statistics. Means were compared by one-way analysis of variance (ANOVA). For statistically significant ANOVA, specific differences among groups were determined by multiple comparison analysis. The Dunnett's multiple comparison test was used when data were compared to a specified control. Otherwise, the Tukey-Kramer multiple comparison test was employed. All analyses were conducted using InStat software (GraphPad Software, San Diego CA).

RESULTS

Gata4 is emerging as an important activator of intestinal genes (4,10,12), but little is known about its importance and underlying mechanism in the activation of the *LPH* gene. To begin to define the importance of Gata4 in the regulation of *LPH* gene expression, the ability of Gata4 from mouse intestine to bind to the GATA binding site in the mouse *LPH* promoter was defined by supershift EMSAs (Fig. 3A). Using nuclear extracts from the epithelial cells of mouse jejunum and the mouse GATA binding site as a probe (mG_1), a specific protein-DNA complex was formed that supershifted with the addition of a Gata4 antibody. Faster mobility complexes which we believe are degradation products of Gata4, also disappeared with the addition of the Gata4 antibody. No supershift was formed using Gata5 or Gata6 antibodies, or with non-immune serum. The antibodies used in this assay recognized their respective antigens, as shown by supershift analysis of nuclear extracts from transfected Caco-2 cells (Fig. 3B). Although other proteins, including Gata4, Hnf1α, β-actin (see below), and Cdx-2 (not shown) are readily detected in these extracts, it remains a possibility that Gata5 and Gata6 are preferentially degraded during nuclear extract isolation. Quantitative analysis from jejunal extracts of four different mice using scanning densitometry revealed that 75% (range, 60-92%) of the complex that formed in the absence of the Gata4 antibody supershifted when the Gata4 antibody was added. These data indicate that Gata4 in nuclear extracts from epithelial cells of the adult mouse jejunum is the primary GATA protein that binds to the GATA site on the mouse *LPH* promoter implicating Gata4 as the principal intestinal GATA regulator of *LPH* gene expression.

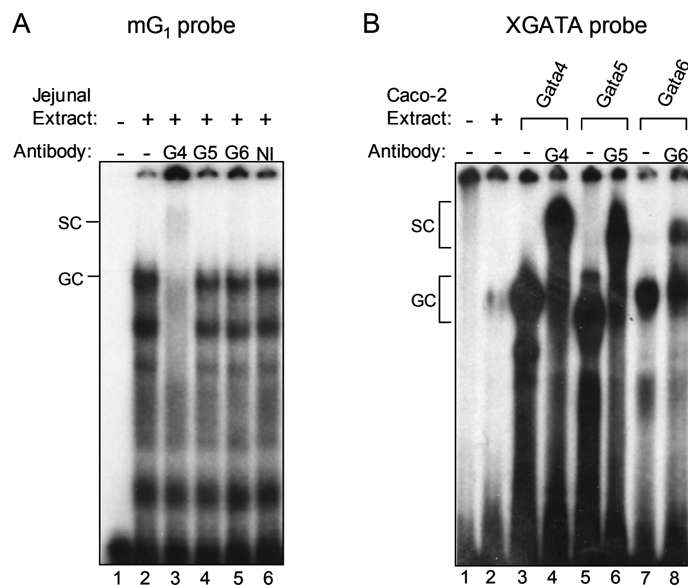

Fig. 3. Gata4 isolated from mouse epithelium binds the GATA site on the mouse *LPH* gene. A. Supershift EMSAs were carried out using nuclear extracts (20 µg) isolated from the epithelial cells of the jejunum of adult mice and the mG_1 site (conserved 5' GATA site at -89 bp) as a probe. A GATA complex (GC) was formed that supershifted (supershift complex, SC) with the anti-Gata4 antibody, but not with the anti-Gata5 or anti-Gata6 antibodies, or non-immune (NI) serum. B. Antibodies used in this study recognize their specific antigens. Supershift EMSAs were carried out using nuclear extracts (10 µg) isolated from Caco-2 cells untransfected (lane 2) or transfected with expression vectors for Gata4 (lanes 3&4), Gata5 (lanes 5&6) or Gata6 (lanes 7&8). The GATA complexes (GC) and supershift complexes (SC) are indicated.

To delineate the function of Gata4 in the regulation of *LPH* gene expression *in vivo*, it is necessary to describe the pattern of Gata4 expression relative to that of *LPH*. Since Gata4 demonstrates cooperativity with Hnf1α (10,73), characterization of the pattern of Hnf1α gene expression was also undertaken. All experiments were conducted in adult mice and focused on the proximal-distal pattern. *LPH* mRNA abundance, as determined by RNase protection assays (Fig. 4A), was highest in the jejunum and proximal ileum, and reduced in the duodenum and distal ileum, corroborating previous results in rodents (6,16,25,27,35,58,69). *Gata4* mRNA abundance was highest in the proximal and middle segments of intestine, and reduced in distal segments, similar to that previously described using Northern analysis (12). Neither *LPH* nor *Gata4* was expressed in colon. *Hnf1α* mRNA abundance was similar throughout the small intestine and colon, as previously shown (60). RNase protection assays also revealed that *Gata5* and *Gata6* mRNAs are expressed in all segments, with *Gata5* being highest in the distal intestine (SD Krasinski, unpublished observation), as previously reported (12). Western analysis (Fig. 4B) demonstrated that the patterns of Gata4 and Hnf1α abundance were correlated with their respective mRNAs. Gata4 was highest in proximal segments and reduced in distal segments, whereas Hnf1α was expressed relatively evenly throughout the mouse small intestine, as previously shown for duodenum and jejunum in 22-day old mice (5). To demonstrate co-expression of Gata4 and Hnf1α in the same cells, and also to confirm the proximal-distal patterns of protein expression, immunohistochemistry on serial sections was carried out on jejunum and ileum (Fig. 4C).

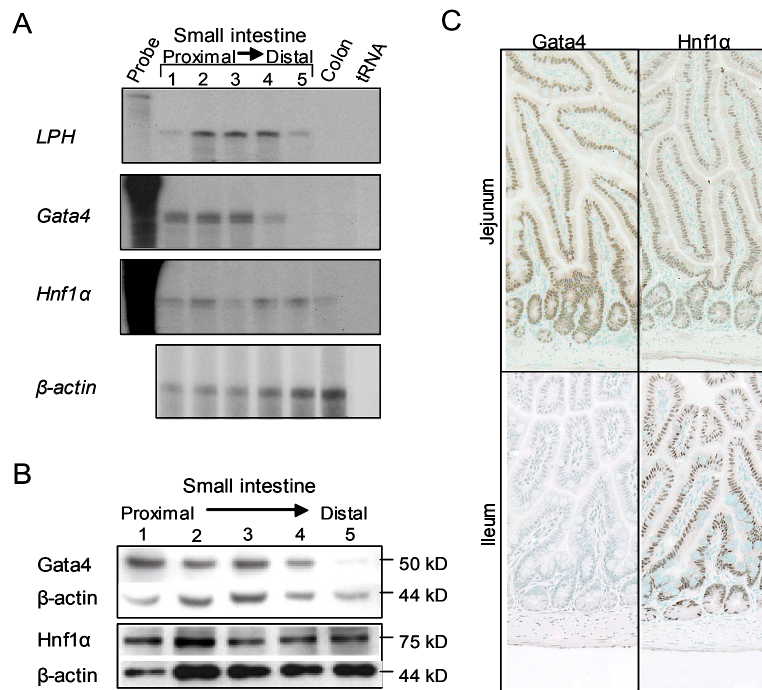

Fig. 4. *LPH* expression is correlated with that of Gata4 in jejunum and ileum, but not in duodenum or crypts. (A). *LPH*, *Gata4*, and *Hnf1α* mRNAs are expressed in distinct patterns along the length of the adult mouse small intestine. RNase protection assays were carried out using 5 μg of total RNA from 5 equidistant segments along the length of the small intestine of an adult mouse as described in Materials and Methods. (B). Gata4 and Hnf1α are expressed in patterns similar to that of their respective mRNAs. Western analysis was conducted on nuclear extracts (20 μg) isolated from 5 segments along the length of the adult mouse small intestine as described in Materials and Methods. (C). Gata4 and Hnf1α are co-expressed in the nuclei of the same cells on intestinal epithelium. Immunostaining for Gata4 and Hnf1α was conducted on serial sections of adult mouse jejunum (segment 2) and ileum (segment 5) using anti-Gata4 and anti-Hnf1α antibodies, as described in Materials and Methods.

In jejunum, Gata4 and Hnf1α were co-expressed in the nuclei of villus enterocytes, in agreement with previous studies (5,10), although we also noted crypt enterocyte staining for both of these proteins. Serial dilutions of both antibodies (not shown) revealed an absence of background at higher concentrations (up to 0.8 μg/ml), and an equal disappearance of crypt and villus staining at lower concentrations (~0.02 μg/ml), verifying the crypt staining. The reason for the differences with published data is unknown, but could be due to differences in sensitivity. In the ileum, Gata4 was not detected, whereas Hnf1α was expressed in all villus enterocytes, in general agreement with RNase protection (Fig. 4A) and Western (Fig. 4B) data. These data indicate that *LPH* expression is correlated with that of Gata4 in jejunum and ileum, but not in duodenum.

Gata4 has already been shown to activate the *LPH* promoter in the Caco-2 intestinal cell line (13,28), but the underlying mechanism has not yet been defined. Thus, transient co-transfection assays using expression vectors for Gata4, Gata5, and Hnf1α were conducted in HeLa cells, which do not synthesize endogenous Hnf1α (28) and are therefore a model for both Hnf1α-independent and cooperative activation. A previously described plasmid containing 118 bp of the human *LPH* promoter, which includes the conserved GATA and HNF-1 binding sites (Fig. 1) fused to the human growth hormone reporter (h118wt) (28,73) was used in these studies. As shown in Fig. 5, Gata5 did not activate the human *LPH* promoter significantly, consistent with previous data in HeLa cells (28), but Gata4 independently activated this promoter over basal expression (>25-fold, $P<0.05$). Both Gata4 and Gata5 synergistically activated the human *LPH* promoter with Hnf1α, as previously shown (73). Gata4 and Gata5 were synthesized in similar amounts as indicated by band densities in EMSAs using the XGATA probe, which binds both proteins equally (17,28,73), and nuclear extracts from transfected HeLa cells (not shown). These data indicate that Gata4 activates the human *LPH* promoter by dual mechanisms, one that is independent of Hnf1α (Gata4-specific activation) and one that demonstrates cooperativity with Hnf1α (GATA/Hnf1α cooperative activation).

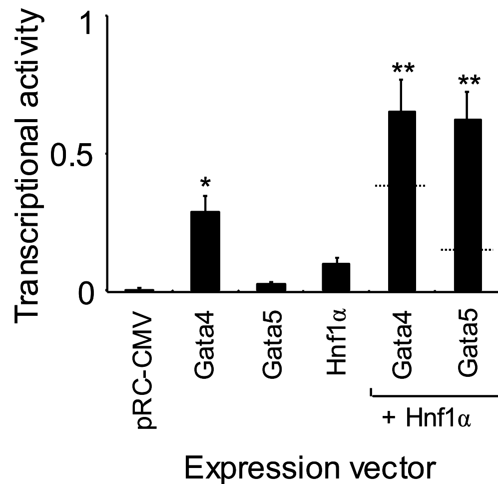

Fig. 5. Gata4 activates the human *LPH* promoter both independently and cooperatively with Hnf1α. A previously described promoter-reporter construct in which 118 bp of the human *LPH* promoter fused to the human growth hormone reporter (28,73) was co-transfected into HeLa cells with pRC-CMV (basal expression) or expression vectors for wild type Gata4, Gata5, or Hnf1α, singly and in combination (indicated along the x-axis). Transcriptional activity was determined as described in Materials and Methods. The dotted line indicates the sum of the transcriptional activities of GATA and Hnf1α expression vectors co-transfected individually. Transcriptional activities above the dotted line indicate synergistic activation. Data are means ± SEM of n = 3. *P<0.05, **P<0.01, as compared to basal (pRC-CMV) expression by Dunnett's multiple comparison test.

To define the underlying mechanisms of each pathway, the requirement of particular structures in Gata4 necessary for Gata4-specific and GATA/Hnf1α cooperative activation was determined by the introduction of mutations into Gata4 that delete or disrupt these structures (Fig. 6). Gata4 is comprised of a pair of N-terminal activation domains (AD), a pair of zinc fingers (Zn) and a basic region (B) that mediate protein-DNA (49) and protein-protein (11,33,37,59,67) interactions, and a C-terminal domain (CTD) of uncertain function. Deletion of the activation domains (-AD) resulted in a 60% reduction in transcriptional activity in the absence of Hnf1α, and a 50% reduction in the presence of Hnf1α, although cooperative activation in the presence of Hnf1α remained above the summation (dotted) line indicating synergy (Fig. 6A). A mutation in a critical cysteine residue (C290S), which disrupts the structure of the C-terminal zinc finger, resulted in the elimination of both Gata4-specific and GATA/Hnf1α-cooperative activation (P<0.05 for each, Fig. 6A). EMSAs (Fig. 6B) and GST pull-down assays (Fig. 6C) indicated that Gata4(-AD) is capable of binding to DNA and physically associating with Hnf1α, respectively, whereas Gata4(C290S) is incapable of binding to DNA (Fig. 6B), as previously shown (49), or physically associating with Hnf1α (Fig. 6C). These data indicate that the C-terminal zinc finger of Gata4 is necessary for Gata4-specific and GATA/Hnf1α-cooperative activation, and is likely due to the inability to bind DNA and physically associate with Hnf1α, respectively.

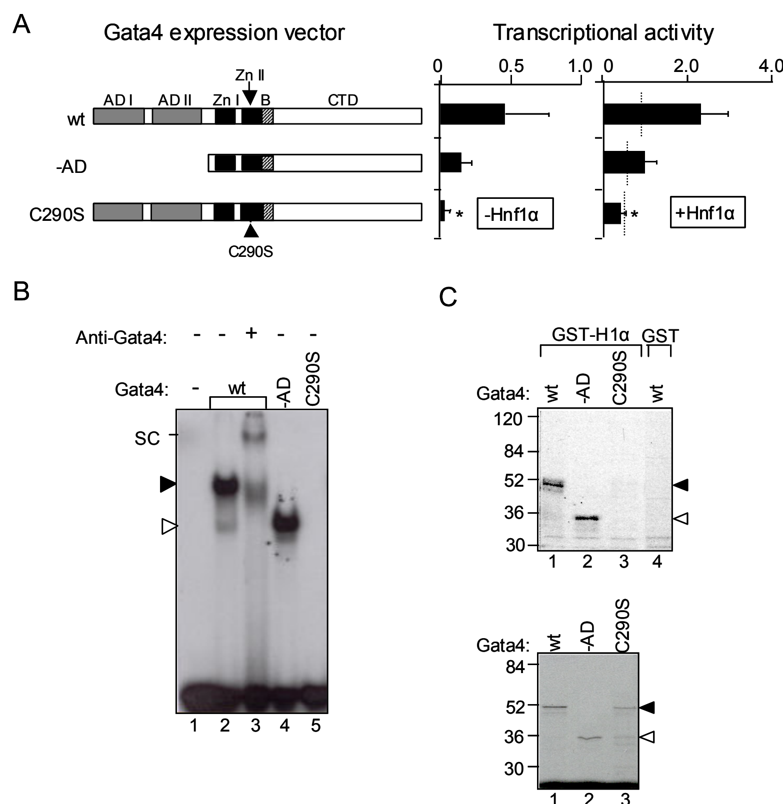

Fig. 6. The C-terminal zinc finger of Gata4 is critical for transcriptional activation. (A). The C-terminal zinc finger is necessary for Gata4-specific and GATA/Hnf1α cooperative activation. HeLa cells were co-transfected with h118wt and wild type or mutated Gata4, in the absence or presence of co-transfected Hnf1α (indicated in the boxes within each bargraph). Gata4(-AD) is an activation domain deletion and Gata4(C290S) contains a cysteine-to-serine substitution in the C-terminal zinc finger. Data are means ± SEM of n = 5. *P<0.05, as compared to co-transfection with wild type Gata4 by Dunnett's multiple comparison test. (B). The C-terminal zinc finger of Gata4 is necessary for DNA binding. EMSAs were carried out using a probe containing a GATA binding site (XGATA) (73) and *in vitro* transcribed and translated wild type and mutated Gata4 proteins, as indicated across the top of the figure. A protein-DNA complex using wild type Gata4 (filled arrowhead, lane 2) forms a supershift complex (SC) with an anti-Gata4 antibody (lane 3). Gata4(-AD) (open arrowhead) binds to DNA, whereas Gata4(C290S) does not bind to DNA. (C). The C-terminal zinc finger of Gata4 is required for physical interaction with Hnf1α. GST pull-down assays (upper panel) were carried out using GST-Hnf1α incubated with labeled *in vitro* transcribed and translated wild type (lane 1) and mutated Gata4 (lanes 2 and 3). Gata4 wt (filled arrowhead) and Gata4(-AD) (open arrowhead) were pulled down by GST-Hnf1α, whereas Gata4(C290S) was not pulled down by GST-Hnf1α. Gata4 was not pulled down by GST alone (lane 4), indicating that Gata4 does not interact with GST. All proteins were synthesized as shown by direct loading of labeled TNT™ products (lower panel).

To define the importance of GATA and HNF-1 binding sites on the *LPH* promoter for Gata4-specific and GATA/Hnf1α cooperative activation, transient co-transfection assays were carried out in HeLa cells using h118 *LPH*-hGH constructs in which the GATA and/or HNF-1 binding sites were mutated (Fig. 7). In a promoter construct in which both GATA sites were mutated, Gata4-specific activation was significantly reduced to less than 20% of that of the wild type promoter (P<0.05), whereas GATA/Hnf1α cooperative activation was reduced to approximately 50% of that of the wild type promoter, but remained above the summation (dotted) line indicating synergy. In a promoter construct in which only the HNF-1 binding site was mutated, Gata4 activation was not compromised, whereas GATA/Hnf1α cooperative activation was reduced to approximately 25% of that of the wild type promoter, and fell below the summation line indicating loss of synergy. In a construct where all three binding sites were

mutated, both Gata4-specific and GATA/Hnf1α cooperative activation were significantly reduced (P<0.05 for each). These findings demonstrate that the GATA binding sites are necessary for Gata4-specific activation, and the HNF-1 binding site is required for GATA/Hnf1α cooperative activation.

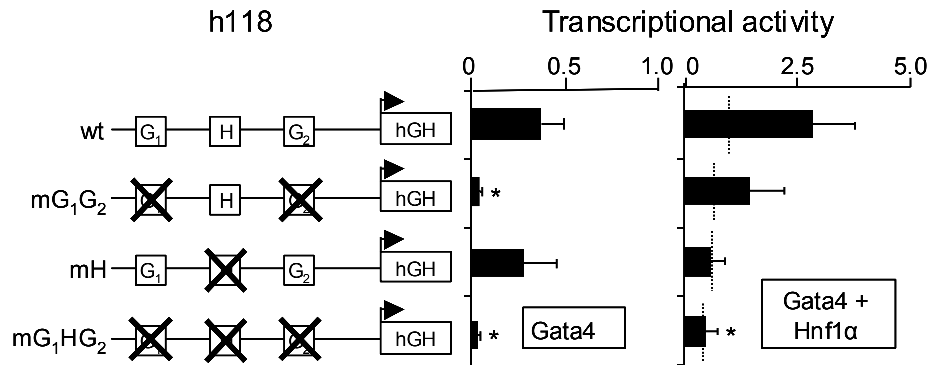

Fig. 7. Different binding sites on the human *LPH* promoter are required for Gata4-specific and GATA/Hnf1α cooperative activation. HeLa cells were co-transfected with wild type and mutated h118 *LPH*-human growth hormone (hGH) promoter-reporter constructs (indicated on the left) and expression vectors for Gata4 alone or Gata4 and Hnf1α together (indicated in the boxes within each bargraph). The h118mG_1G_2, h118mH, and h118 mG_1HG_2 constructs contain mutations introduced into both GATA binding sites, the HNF-1 site, and all three sites together, respectively. These mutations have been previously shown to disrupt specific protein-DNA interactions (28). Data are means ± SEM of n = 5. *P<0.05, as compared to h118wt by Dunnett's multiple comparison test.

Since binding of Gata4 to DNA is a requirement for Gata4-specific activation (Fig. 7), and since the zinc finger and basic regions of GATA factors mediate DNA binding (42,49,73), we next tested the hypothesis that the zinc finger and basic regions are responsible for the differential abilities of Gata4 and Gata5 to activate the *LPH* promoter in absence of Hnf1α. In these studies, expression vectors were constructed in which the zinc finger and basic regions of Gata4 and Gata5 were interchanged and tested for their ability to activate the *LPH* promoter in HeLa cells. Gata4(ZnG5), which contains the activation and C-terminal domains of Gata4, but the zinc finger and basic region of Gata5, failed to activate the *LPH* promoter (Fig. 8A).

In contrast, Gata5(ZnG4), which contains the activation and C-terminal domains of Gata5, but the zinc finger and basic region of Gata4, demonstrated an activity that was similar to that of wild type Gata4. These expression vectors synthesized similar amounts of GATA protein, as shown by EMSA using nuclear extracts from transfected HeLa cells and the XGATA probe (Fig. 8B). These proteins were all capable of binding GATA sites on DNA (Fig. 8B), were recognized by epitope-specific antibodies (supershift, not shown), and synergistically activated

the *LPH* promoter with Hnf1α (>3-fold synergy for all, not shown), indicating that the proteins were structurally intact. These findings reveal that Gata4-specific activation of the *LPH* promoter is mediated by the zinc finger and basic regions.

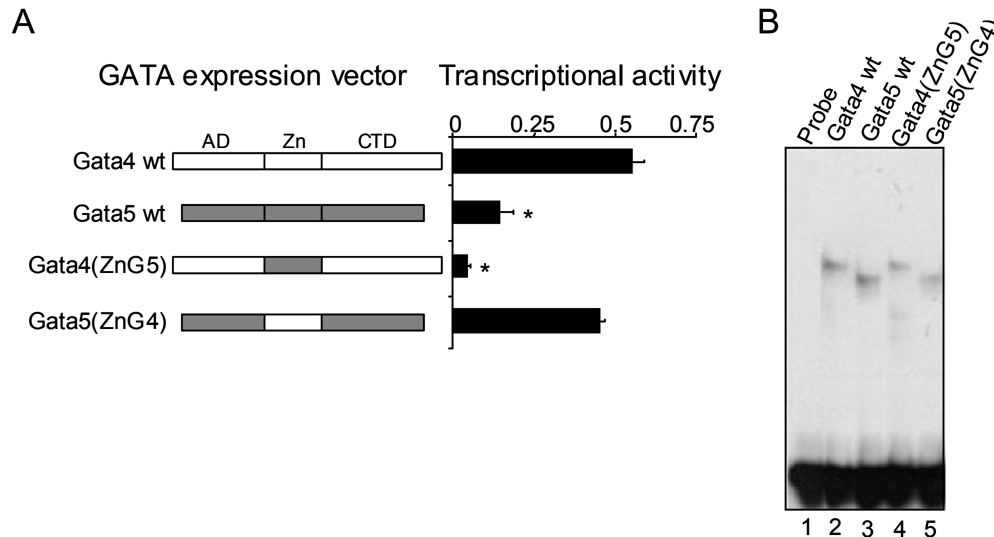

Fig. 8. Gata4-specific activation is mediated by the zinc finger and basic regions. (A) Heterologous Gata5 containing the Gata4 zinc finger region demonstrates Gata4-specific activation. HeLa cells were co-transfected with h118wt and wild type or heterologous Gata4 or Gata5 containing zinc finger swaps as indicated on the left. Data are means ± SEM of n = 4. *P<0.01, as compared to Gata4 wt and Gata5(ZnG4) by the Tukey-Kramer multiple comparison test. (B) Mutated GATA factors bind GATA sites on DNA. EMSAs carried out using nuclear extracts from transfected HeLa cells (5 μg) and the XGATA probe reveal similar intensities of specific complexes.

Since the zinc finger and basic regions of GATA factors mediate binding to DNA (42,49,73), we hypothesized that the differences in the ability of Gata4 and Gata5 to activate the *LPH* promoter in the absence of Hnf1α is due to a greater affinity for the GATA binding sites on the human *LPH* promoter by Gata4 as compared to Gata5. To test this hypothesis, a series of affinity EMSAs were conducted (Fig. 9). In the first experiment, competition EMSAs were carried out in which the ability of the GATA binding sites on the *LPH* promoter to preferentially compete Gata4 or Gata5 away from a standardized GATA binding site was determined (Fig. 9A). Previous studies indicated that the GATA binding site in the Xenopus *Fabp2* gene (i.e., XGATA) binds both Gata4 and Gata5 similarly (17,28,73), and thus was used as the standard control probe. Gata4 and Gata5 are different in size (48), and thus can be added together and analyzed simultaneously. Supershift analysis verified that the slower mobility complex was Gata4 (lane 3) and the faster mobility complex was Gata5 (lane 4). XGATA competed both bands similarly (lanes 5-7), as anticipated. The hG_1 and hG_2 oligonucleotides also competed each band relatively equally, suggesting that they bind Gata4 and Gata5 with similar affinities. In the

second experiment, affinity was tested directly as previously described (7) using constant amounts of synthetic Gata4 or Gata5 and limiting amounts of probe (Fig. 9B). Probe titrations revealed no difference in complex intensities between Gata4 and Gata5 for both hG_1 and hG_2. Together, these data indicate that the GATA sites on the human *LPH* promoter bind Gata4 and Gata5 with similar affinities, and thus do not account for the differences in the ability of these two transcription factors to activate the *LPH* promoter in co-transfection experiments. Experiments to identify alternative mechanisms underlying these differences are in progress in our laboratory.

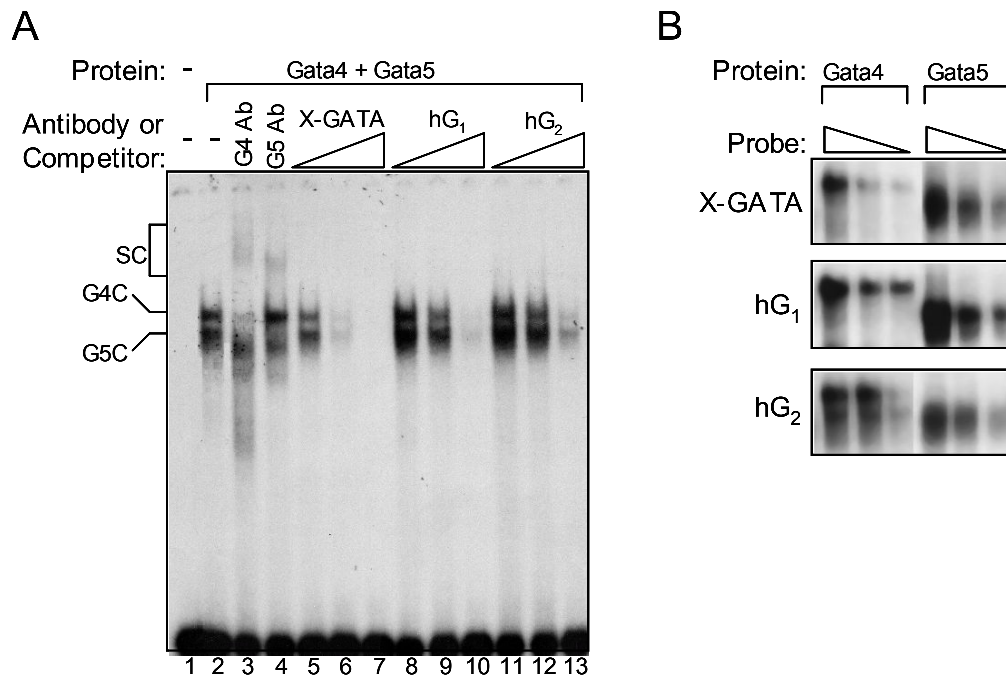

Fig. 9. Gata4 and Gata5 have similar affinities for the GATA binding sites on the human *LPH* promoter. (A) EMSAs showing similar competition of Gat4 and Gata5 complexes by the GATA binding sites in the *LPH* promoter. Using the XGATA probe and a mixture of *in vitro* transcribed and translated Gata4 and Gata5 proteins (lanes 2-13), competition EMSAs were carried out with titrated amounts of oligonucleotide competitors (5-, 20-, 100-fold molar excess), including XGATA (lanes 5-7), hG_1 (lanes 8-10) and hG_2 (lanes 11-13). Supershift complex (SC) formation confirmed that the band with the slower mobility was the Gata4 complex (G4C, lane 3) and that with the faster mobility was the Gata5 complex (G5C), as anticipated based on the size of the proteins. (B) EMSAs showing similar affinities of Gata4 and Gata5 for the binding sites on the *LPH* promoter. Constant amounts of synthetic Gata4 or Gata5 were incubated with limiting amounts of XGATA, hG_1, and hG_2 probes.

DISCUSSION

Intestinal differentiation occurs as a result of a precisely orchestrated program involving complex interactions among general as well as tissue-restricted transcription factors and co-factors. Markers of intestinal differentiation, namely those genes whose expression is confined to the differentiated villus epithelium, are used as models to study the underlying mechanisms that direct this critical process (68). *LPH* is an excellent model for the study of intestinal differentiation because in small intestine of adult mammals it is expressed only in absorptive enterocytes on villi in a precisely regulated pattern along the proximal-distal axis (6,16,25,27,35,45,52,57,58,69). In the present study, we show that Gata4 is the principal GATA factor that binds to the *LPH* promoter (Fig. 3), and correlates well with *LPH* mRNA in jejunum and ileum (Fig. 4), implicating Gata4 as the primary GATA regulator of *LPH* gene expression *in vivo*. Gata4 synergistically activates the *LPH* promoter by a mechanism identical to that previously characterized for Gata5/Hnf1α (73) suggesting a conserved pathway among intestinal GATA factors. Gata4 also activates the *LPH* promoter by a mechanism that is distinct from that of Gata5, demonstrating for the first time independent functions among individual intestinal GATA factors.

Gata4 is emerging as the principal GATA regulator of intestinal genes. Gata4 was the primary GATA factor in nuclear extracts isolated from adult mouse jejunum that binds the GATA sites in the *SI* (4), *ADA* (12), *Fabp2* (9), and *LPH* (Fig. 3) promoters. In all of these studies, binding of Gata5 and Gata6 could not be detected. Although *Gata4*, *-5*, *-6*, mRNAs are all expressed in the intestine of adult mice (SD Krasinski, unpublished observation) (12), only Gata4 and Gata6 protein have thus far been detected in post-weaning mice (4,10,12) (Fig. 3 & 4). Indeed, intestinal Gata5 could not be detected by immunostaining in spite of positive control immunostaining of Gata5 in stomach (10), although its expression could be below the detection limits of the assay. Gata6 is present in adult mouse epithelium as shown by immunostaining (10), but binding to intestinal gene promoters was not detected by us (Fig. 3) or others (4,10,12). Gata6 was found to bind the *treh* promoter in 8 day old mice (53). Thus, it is possible that Gata5 and/or Gata6 are critical for *LPH* gene expression at developmental time-points other than adults. In chimeric mice harboring intestinal epithelial cells that do not express Gata4, Divine et al (9) showed that *Fabp2* expression was absent as compared to wild-type cells in the same animal, providing key *in vivo* data demonstrating a critical importance of Gata4 for intestinal *Fabp2* expression, and a lack of overlapping functions with other intestinal GATA factors. Thus, although all three members of the Gata4, -5, -6 subfamily activate intestinal gene promoters in over-expression experiments in cell culture models (9,13,17,22,28,73), data in adult mice support a principal role for Gata4 as the key GATA regulator of intestinal gene expression *in vivo*.

LPH gene expression in the adult mouse small intestine is highly correlated with that of Gata4 in the jejunum and ileum, but not in the duodenum (Fig. 4). In the jejunum and proximal ileum, *LPH* and Gata4 are both expressed at high levels, and in the distal ileum, *LPH* and Gata4

are both expressed at reduced levels, consistent with the hypothesis that Gata4 is an activator of *LPH* gene expression in these regions. In the duodenum where *LPH* expression is low, Gata4 expression is high. Reduced *LPH* gene expression in the duodenum could be due to a reduction or modification of a critical activator, or to activation of a repressor pathway. Hnf1α, a proposed activator of the *LPH* promoter (28,40,41,64), is expressed equally throughout the small intestine (Fig. 4), as previously shown (60), and is thus unlikely to account for differential *LPH* expression along the proximal-distal axis. Cdx2, also a proposed activator of the *LPH* promoter (14,28,41,71,72,74), is reduced in proximal vs. distal intestinal segments (5,61), and thus may be responsible for the reduced duodenal expression of *LPH*. It is also possible that *LPH* is specifically repressed in the duodenum. Preliminary data suggest that Pdx1, whose intestinal expression is confined to the duodenum (19), represses *LPH* gene expression in this region of small intestine (76). Precedence for a repressor pathway is supported by Cux/CDP, a homeodomain-containing transcriptional repressor closely related to the *cut* protein in *Drosophila melanogaster*, which represses *SI* gene expression in distal ileum and colon, as shown by ectopic ileal and colonic *SI* expression in mice that do not express Cux/CDP (3). Together, these data support the hypothesis that the pattern of *LPH* gene expression along the proximal-distal axis is defined, in part, by Gata4, but that other pathways are also involved.

Gata4 may activate the *LPH* promoter in concert with Hnf1α through an evolutionarily conserved mechanism. Serial sectioning experiments (Fig. 4) demonstrate that Gata4 and Hnf1α are co-expressed in the same absorptive enterocytes on villi, as previously indicated (4,10), providing the topographic basis for molecular interactions. Indeed, *LPH* expression in adult mice always occurs in regions that co-express Gata4 and Hnf1α, although *LPH* is not always expressed where Gata4 and Hnf1α are co-expressed, such as in crypts, suggesting that Gata4 and Hnf1α are necessary, but not sufficient for *LPH* gene expression. Preliminary data in our laboratory using germline *hnf-1α* null mice demonstrate ~90% reduction in *LPH* gene expression in adult jejunum (26), supporting an important role for Hnf1α as an activator of *LPH* gene expression *in vivo*. In cell culture models, Gata4 (Fig. 5 & 6) and Gata5 (73) physically associate with Hnf1α to synergistically activate the *LPH* promoter, and all three GATA factors interact with Hnf1α to synergistically activate the *Fabp2* promoter (9). Gata4, Hnf1α, and Cdx2 may all cooperate together in the activation of the *SI* promoter (4,28). For both Gata4 and Gata5, physical association with Hnf1α is mediated by their C-terminal zinc fingers and basic regions (73) (Fig. 6). Binding sites on the *LPH* promoter for Hnf1α, but not for GATA, are necessary for both Gata4/Hnf1α and Gata5/Hnf1α synergy (73) (Fig. 7). Together, these studies suggest that cooperative interaction with Hnf1α is a conserved pathway among intestinal GATA factors for the activation of intestinal genes.

Gata4 and Gata5 also demonstrate differential regulation of the *LPH* promoter in that in the absence of Hnf1α, Gata4 is capable of activating the *LPH* promoter alone, but Gata5 is not (Fig. 5). Differences in the ability of individual members of the Gata4, -5, -6 subfamily to activate target genes have been previously reported for non-intestinal genes. Using affinity

EMSAs performed with increasing amounts of radiolabeled probes and a constant amount of protein as was conducted in the present investigation (Fig. 9), Charron et al (7) showed that preferential regulation of the α-myosin heavy chain (MHC) gene by Gata4 was due in part to a greater affinity of Gata4 compared to Gata6 for the GATA site on the α-MHC promoter. In contrast, Morrissey et al (50) found that when the zinc finger DNA binding domains of Gata4 or Gata6 were fused to the VP16 activation domain, a *Dab2* promoter-reporter construct containing a GATA-binding site was activated equally indicating that differential regulation was not due to the zinc fingers, and therefore, unlikely to be due to differences in DNA binding affinity. Using the differences in the capacity of Gata4 and Gata5 to activate the *LPH* promoter in the absence of Hnf1α as a model in structure-function studies involving a domain swapping strategy, we determined that Gata4-specific activation of the *LPH* promoter is mediated by the zinc finger and basic regions (Fig. 8). However, although the zinc finger/basic regions mediate DNA binding, Gata4 and Gata5 surprisingly demonstrate virtually identical affinities for the binding sites on the *LPH* promoter (Fig. 9) suggesting that the underlying differences in the ability of Gata4 and Gata5 to activate the *LPH* promoter is not due to differences in affinity for binding to DNA. Thus, GATA subfamily members demonstrate multiple pathways for specific regulation of target genes.

Several issues remain a challenge for future studies. First, the contribution of GATA/Hnf1α cooperativity vs. Gata4-specific activation of *LPH* gene expression *in vivo* remains to be determined. Although Gata4 and Hnf1α are co-expressed in the same cells (Fig. 4), and synergistically activate intestinal gene promoters in over-expression experiments in cell culture assays (9,28,73), the importance of Gata4/Hnf1α cooperativity at stoichmetric levels *in vivo* for intestinal gene expression remains unknown. Second, the mechanisms underlying the complex topographic patterns of intestinal gene expression are yet to be defined. Although *LPH* expression occurs where Gata4 and Hnf1α are highly co-expressed, *LPH* expression is low or absent in duodenum and in crypts (Fig. 4), respectively, where Gata4 and Hnf1α are also highly co-expressed implicating the involvement of other pathways. Finally, the mechanisms underlying the differential functions of Gata4 and Gata5 remain to be determined. We mapped the functional components to the zinc finger/basic regions of Gata4 and Gata5 (Fig. 8), but showed that the differential function was not due to differences in binding affinity to the *LPH* promoter (Fig. 9). Alternative mechanisms for all of these issues include the participation of other transcription factors and/or other proteins that may act as co-activators or co-repressors, as well as modification pathways that could alter protein function. Other transcription factors and co-factors include Cdx2 (40,41,64,74), SP1 (22,38), HoxC11 (40), friend of GATA (FOG) (37,67), and the dimerization co-factor of Hnf1α (DcoH) (39). GATA subfamily members and Hnf1α may also recruit coactivators such as CBP and p300/CBP-associated factor (20,63), forming a complex that activates transcription by coupling nucleosome modification with recruitment of proteins for the general transcription machinery. Gata4 may be directly modulated by phosphorylation (23,36,46) or acetylation (77), which may influence both DNA binding affinity

and trans-activation potential. Other information further upstream in the *LPH* promoter (8,35,64,70) may also be important to interpret *in vivo LPH* gene expression. The future challenge will be to delineate the combinatorial roles of Gata4 and Hnf1α, and their interaction with other factors and cofactors, as well as their possible modification, in the spatial and temporal regulation of intestinal differentiation.

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CHAPTER 3

Hepatocyte nuclear factor-1alpha is required for gene-expression, but dispensable for histone acetylation of the lactase-phlorizin hydrolase gene

Tjalling Bosse[1], Herbert M. van Wering[2], Marieke Gielen[1], Lauren N. Dowling[3], John J. Fialkovich[3], Christina Piaseckyj[3], Frank J. Gonzalez[4], Taro E. Akiyama[5], Robert K. Montgomery[3,6], Richard J. Grand[3,6], Stephen D. Krasinski[3,6,7]

From the [1]Department of Pediatrics, University of Amsterdam, Amsterdam, The Netherlands 1100DD; and [2]Department of Medicine, Free University of Amsterdam, Amsterdam, The Netherlands 1081HV; and [3]Division of Gastroenterology and Nutrition, Department of Medicine, Childrens Hospital, Boston, Massachusetts, 02115; and [4]National Cancer Institute, National Institute of Heath, Bethesda, MD 20892-0001; and [5] Merck Research Laboratories, Rahway, NJ 07065; and [6]Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, 02115; and [7]Dorothy R. Friedman School of Nutrition Science and Policy, Tufts University, Boston, Massachusetts, 02111

ABSTRACT

Hepatocyte nuclear factor-1alpha (Hnf1α) is a modified homeodomain-containing transcription factor that has been implicated in the regulation of intestinal genes. To define the importance and underlying mechanism of Hnf1α for the regulation of intestinal gene expression *in vivo*, we analyzed the expression of the intestinal differentiation markers and putative Hnf1α targets, lactase-phlorizin hydrolase (LPH) and sucrase-isomaltase (SI), in *Hnf1α* null mice. We found that in adult jejunum, *LPH* mRNA in *Hnf1α* -/- mice was reduced 95% as compared to wild-type controls ($P<0.01$, $n=4$), whereas SI mRNA was virtually identical to that in wild-type mice. Further, *SI* mRNA abundance was unchanged in the absence of Hnf1α along the length of the adult mouse small intestine as well as in newborn jejunum. We found that Hnf1α occupies the promoters of both the *LPH* and *SI* genes *in vivo*. However, in contrast to liver and pancreas where Hnf1α regulates target genes by recruitment of histone acetyl transferase activity to the promoter, the histone acetylation state of the *LPH* and *SI* promoters was not affected by the presence or absence of Hnf1α. Finally, we show that a subset of hypothesized intestinal target genes are regulated by Hnf1α *in vivo*, and this regulation occurs in a defined tissue-specific and developmental context. These data indicate that Hnf1α is an activator of a subset of intestinal genes, and induces these genes through an alternative mechanism in which it is dispensable for chromatin remodeling.

INTRODUCTION

Hepatocyte nuclear factor-1α (Hnf1α), a modified homeodomain-containing transcription factor originally identified in liver by its ability to interact with regulatory elements in the albumin and β-fibrinogen genes (7, 8), has since been implicated in the regulation of gastrointestinal genes. Hnf1α is expressed in liver, pancreas and kidney (2,3), as well as in the epithelium of the stomach, small intestine and colon (19). In the small intestine of adult rodents, Hnf1α is expressed evenly from duodenum to ileum in the proliferative crypt compartment as well as in differentiated cells on villi (4, 39, 49). It is expressed in the small intestine throughout development (4, 39). Hnf1α binds *in vitro* to specific regulatory regions in multiple genes expressed in the small intestinal epithelium (4,6, 9, 11, 12, 15, 18, 20, 23, 27, 37, 38, 43, 52, 54), and has been shown to activate the promoters of most of these genes in over-expression experiments in cell culture transient co-transfection assays (4, 6, 9, 14, 15, 18, 37, 52, 54). In germline *hnf1α* null mice, the mRNAs for the cystic fibrosis transmembrane conductance regulator (*CFTR*), *Claudin-2* and *calbindin D9k* were modestly reduced (~50%) (27, 37, 52), whereas that of the apical sodium-dependent bile acid transporter (*ASBT*) was silenced (40). In transgenic mice expressing reporters under the control of the sucrase-isomaltase (*SI)* or liver fatty acid binding protein (*Fabp1)* promoters, mutations in well characterized HNF-1 binding sites resulted in significant reductions in transgene expression (6, 10). Hnf1α may interact with other intestinal transcription factors, including Hox C11 (24), Cdx-2 (4, 18, 25), and GATA factors (4, 10, 18, 49, 50). Taken together, these data suggest that Hnf1α controls the expression of multiple intestinal genes through direct binding to the promoter, and likely regulates these genes through cooperation with other transcription factors.

Lactase-phlorizin hydrolase (LPH) and SI are absorptive enterocyte-specific, microvillus membrane disaccharidases necessary for the terminal digestion of certain carbohydrates in mammals: LPH hydrolyzes the β-linkage in lactose, whereas SI hydrolyses the α-linkage in α-dissacharides (13). The genes encoding these disaccharidases are widely used as models to study intestine-specific gene expression and intestinal differentiation (16, 17, 35, 36, 44). In adult mammals, *LPH* and *SI* expression is confined to absorptive enterocytes on villi, and is highest in the jejunum and proximal ileum, although *SI* is also expressed in the distal ileum (16, 36). *LPH* is highly expressed just prior to birth through suckling and declines at weaning, whereas *SI* is low prior to weaning and increases during weaning. Although this pattern coincides with a change from a milk-based diet to a diet of solid foods containing α-disaccharides, it is well known that *LPH* and *SI* are not regulated by their substrates (13). *LPH* and *SI* have well characterized HNF-1 binding sites in their 5'-flanking regions, called *cis*-element-2c (CE-2c) in *LPH* (47) (-73 to -61 in the mouse *LPH* promoter) and SI footprint 3 (SIF3) in *SI* (55) (-174 to -155 bp in the mouse *SI* promoter). These sites mediate Hnf1α activation in transient cotransfection assays in cell culture models (4, 18), and SIF3 is necessary *in vivo* for the expression of a transgene under the control of the *SI* promoter (6). However, the association of

Hnf1α with CE-2c or SIF3 of the endogenous gene *in vivo* has not yet been reported. We have shown that Hnf1α interacts with GATA factors, namely Gata4, to cooperatively activate the *LPH* and *SI* promoters (4, 18, 49, 50) through an evolutionarily conserved mechanism that requires the DNA binding and activation domains of Hnf1α, and the HNF-1 binding sites on target promoters (49, 50). Together, these data support a role for Hnf1α as a transcriptional activator of *LPH* and *SI* gene expression, and validate the use of *LPH* and *SI* as models for Hnf1α regulation in the mammalian small intestine.

Targeted disruption of *hnf1α* has been reported by two laboratories from which the importance and underlying mechanism of Hnf1α regulation is beginning to be elucidated (21, 31). *Hnf1α* null mice display delayed growth, liver dysfunction, diabetes and sterility, but there is no structural defect in the intestine (40). In these mice, hepatic expression of phenylalanine hydroxylase (*pah*) and *Fabp1* was markedly reduced (1, 32), whereas the expression of *pah* in the pancreas was unaffected (29). In contrast, hepatic expression of the glucose transporter 2 (*glut2*) and L-type pyruvate kinase (*L-PK*) genes was expressed normally in *hnf1α* null mice, but markedly attenuated in pancreas. These findings reveal a differential regulation of target genes in liver and pancreas by Hnf1α (29). Although Hnf1α was associated with these promoters in all tissues where these genes are expressed, Hnf1α was indispensable for transcriptional activation only in cellular- and promoter-specific contexts in which it was required to recruit histone acetyl transferase (HAT) activity (29). Whether Hnf1α is required for the recruitment of HAT activity in the activation of intestinal genes is unknown.

In the present study, we hypothesized that Hnf1α is required for the expression of the *LPH* and *SI* genes as well as other putative Hnf1α targets *in vivo*, and activates these genes by mediating local histone acetylation at the promoters. In our experimental model, we used mice that do not express *hnf1α* rather than by relying on cell culture models dependent on over-expression of transcription factors and extra-chromosomal promoter reporter plasmids. We found that Hnf1α is essential for the expression of the *LPH* gene, but is not necessary for that of *SI*. We further show that Hnf1α is associated with the binding sites on the *LPH* and *SI* promoters *in vivo*, but this interaction does not necessarily correlate with transcriptional activation, as has been shown for genes expressed in liver and pancreas (29). Finally, we demonstrate that the histone acetylation state of the *LPH* and *SI* promoters is not affected by the presence or absence of Hnf1α suggesting that in contrast to liver and pancreatic genes (29), Hnf1α is not required for histone acetylation and subsequent activation of specific intestinal targets. These data are consistent with the hypothesis that Hnf1α regulates a subset of intestinal genes by a mechanism in which it is dispensable for chromatin modification.

MATERIALS AND METHODS

Mice. Mice segregating a null *hnf1α* allele on a C57BL/6J background were generated by deletion of the first exon using Cre-LoxP technology (21). Mice were housed under standard conditions in the Animal Research at Children's Hospital (ARCH) facility and provided food and water *ad libitum*. To identify *hnf1α* wild-type and null alleles, DNA was obtained from tail snips, and a 3-primer PCR strategy was employed (T. Akiyama, unpublished). Absence of Hnf1α expression in the intestine of *hnf1α*-/- mice was confirmed by Western analysis and immunohistochemistry as described (49). To obtain tissue for study, mice were anesthetized and tissue was extracted through a midline incision. All adult study animals were 8-16 weeks of age, and all tissue was collected between 1300 and 1600 hr to avoid any fluctuations in gene expression due to circadian cycles (34). Approval was obtained from the Institutional Animal Care and Use Committee for all experiments involving mice. *RNA isolation.* RNA was isolated from 30-50 mg of mouse small intestine, colon and liver 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 37oC 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.

RNase protection assays. To determine the effect of the absence of *hnf1α* on *LPH* and *SI* mRNA abundance, RNase protection assays were conducted as described (16, 17). To construct a plasmid template for the synthesis of an antisense mouse *LPH* RNA probe, mouse *LPH* cDNA sequence (+285 to +535bp) was amplified and subcloned into pBluescript II KS(+). The plasmid was linearized with *XbaI* and transcribed using SP6 RNA polymerase. The template for a mouse *SI* probe was kindly provided by Dr. P. Traber (University of Pennsylvania) (22). A mouse *β-actin* probe (17) was used as a control for tissue RNA. Gel-isolated [32P]-labeled probes were hybridized to RNA at 68oC in 50% formamide overnight, digested with RNase A and T1, and the protected fragments were separated on 6% denaturing polyacrylamide gels and revealed by autoradiography.

Real-time quantitative RT-PCR (qRT-PCR). To quantify *LPH* and *SI* mRNA levels in wild-type and *hnf1α* null mice, real-time qRT-PCR was performed using an iCycler and iQ SYBR Green Supermix (Biorad). Specific primer pairs (Fig. S1) were designed using Beacon Designer software (Biosoft International), which enables selection of primers that are highly specific for intended targets, and do not form primer-dimers. Temperature gradient and melt curves were obtained for all primer pairs to define optimal cycling conditions and to confirm the absence of primer-dimer formation, respectively. All reactions were conducted in triplicate using 10 pmol of both forward and reverse primers at an annealing temperature of 59ºC and an extension temperature of 72ºC. To calculate the efficiency of the PCR reaction, a standard curve was produced using 10-fold dilutions of calibrator RNA. Calibrator RNA was a pool of intestinal RNA obtained from wild-type adult mouse jejunum. RNA abundance was corrected for *gapdh*

and expressed relative to the calibrator using the Pfaffl method (30), which takes into account the efficiency of amplication of each primer pair. No-RT controls were used for all samples to confirm the absence of DNA contamination. Significant differences in mRNA abundance between *hnf1α* wild-type and null mice were determined by the student t test.

Semi-quantitative RT-PCR. To define the effects on other transcriptional regulators and potential intestinal Hnf1α targets, semi-quantitative RT-PCR was performed on total RNA from jejunum and duodenum of wild-type and *hnf1α* null mice. Primers as shown in Fig. S1 were designed with Beacon Designer software as described above. Optimal temperature and cycling conditions in the linear range were determined for each primer set with wild-type cDNA using an iCycler (Biorad). cDNA was prepared with RNA (0.8μg) from two wild-type and two *hnf1α* null mice using the iScript cDNA synthesis kit (Biorad) following the manufacturer's instructions. For each reaction, 2μl of cDNA was used in a 25μl PCR reaction. The PCR products were separated on a 2% agarose gel.

Electrophoretic mobility shift assays (EMSAs). To define protein-DNA interactions, EMSAs were carried out as previously described (18) using probes and/or competitors shown in Fig. S1, and nuclear extracts from isolated epithelial cells (49). For supershift analyses, antibodies (0.1 μg/μl) were pre-incubated with the nuclear extracts for 20 min prior to the addition of the probe. The antibodies used in EMSAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) as the concentrated gel shift stock (Hnf1α, sc-6547X; Hnf1β, sc-7411X). Both antibodies were verified by the specific supershift complex produced in control experiments using *in vitro* transcribed and translated proteins. All experiments were conducted on at least three different animals.

Chromatin Immunoprecipitations (ChIP) assays. To analyze the association of Hnf1α and the acetylated forms of histone 3 and histone 4 with the mouse *LPH* and *SI* promoters, ChIP assays were performed by a procedure adapted from protocols used for mammalian cells (29, 42). The small intestine of anesthetized mice was removed and placed on a glass plate on a bed of ice, rinsed with ice cold 1X PBS and 5cm of midjejunum was isolated and opened longitudinally. Epithelial cells were collected by scraping the mucosa with glass microscope slides, washed in 1X PBS, and cross-linked for 10 min at room temperature in a final concentration of 1% formaldehyde (Sigma) in 1XPBS. Fixation was terminated by replacing the formaldehyde solution with 0.125 M glycine in 1X PBS. Cells were collected and washed with ice cold 1X PBS and allowed to swell on ice for 10 min in lysis buffer (25mM Hepes, pH 7.8, 1.5mM MgCl2, 10 mM KCl, 0.1% NP-40, 1mM DTT and protease inhibitor cocktail (Sigma)). Following homogenization with a Dounce homogenizer (10 strokes), the nuclei were collected by centrifugation, resuspended in sonication buffer (50mM Hepes, pH 7.8, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and protease inhibitors) and sonicated on ice (10 times 20 sec pulses, Ultrasonics, model W-220F) until the chromosomal DNA ranged from 0.1-1.0 kb in length. Cellular debris was removed by centrifugation at 14K rpm, and the supernatant containing the fragmented chromatin was precleared with protein-G-

Sepharose (Active Motif). Immunoprecipitation was conducted on the precleared chromatin (20μg) using 7.5μl of anti-Hnf1α antibody (sc-6547, SantaCruz), or 4μl of anti-acetylated histone H3 or anti-acetylated histone H4 antibodies (06-599, 06-866, Upstate). The immune complexes were purified by adsorption to protein-GSepharose beads, which were washed once with sonication buffer, once with sonication buffer containing 500mM NaCl, once with 20mM Tris, pH 7.8, 1mM EDTA, 250mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, and twice with Tris-EDTA (TE) buffer. SDS was omitted in the wash buffers for the Hnf1α immunoprecipitations. The beads were incubated in elution buffer (50mM Tris, pH 8.0, 1mM EDTA and 1% SDS) at 65oC for 15min, then elution buffer containing 200mM NaCl at 65oC for 4 h to reverse the formaldehyde cross-links. Samples were then treated with 1μg of proteinase K (25mg/ml)for 1 h at 45oC followed by purification with PCR purification columns (Qiagen). PCR reactions were performed in a 25μl reaction volume containing 2μl of immunoprecipitate or input DNA (fragmented, reverse cross-linked chromatin), and primer mixtures designed to amplify segments containing the transcription initiation site and mCE-2c for *LPH* or mSIF3 for *SI*. Amplification of *LPH* or *SI* coding regions was used as negative controls.

RESULTS

LPH mRNA is reduced in hnf1α null mice – Based on *in vitro*, cell culture, and transgenic data, Hnf1α is hypothesized to be an activator of the *LPH* and *SI* genes in vivo. To test this hypothesis, the abundance of *LPH* and *SI* mRNAs in the adult jejunum of *hnf1α* null mice was compared to that in heterozygous or wild-type littermates. As shown in Fig. 1A, *LPH* mRNA was greatly reduced in an *hnf1α*-/- mouse as compared to an *hnf1α*-/+ littermate using RNase protection assays, whereas *SI* mRNA was not affected by the absence of Hnf1α.

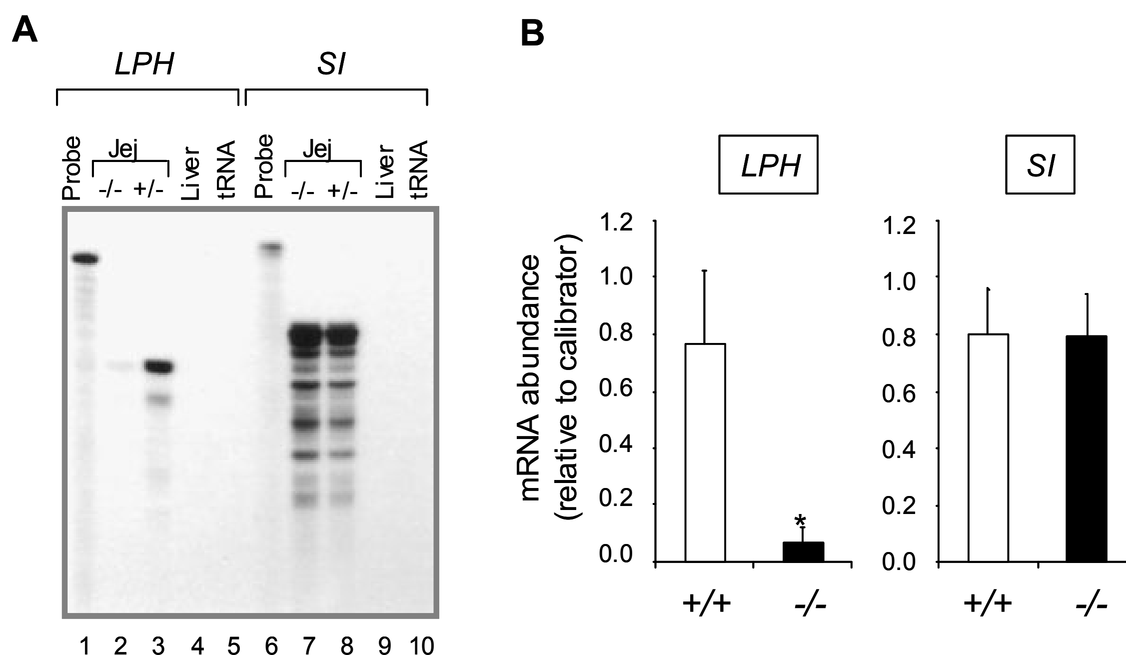

Fig. 1. *LPH* mRNA abundance is reduced in the absence of Hnf1α. (A) RNase protection assays using probes specific for mouse *LPH* (lane 1-5) and *SI* (lane 6-10) mRNAs were carried out on RNA isolated from the jejunum of mice homozygous (-/-, lanes 2&7) and heterozygous (-/+, lanes 3&8) for the *hnf1α* null allele. Mouse liver RNA (lanes 4&9) and tRNA (lanes 5&10) were used as negative controls. (B) Real-time qRTPCR of *LPH* and *SI* mRNA from jejunum revealed a 95% reduction in *LPH* mRNA abundance in the null mice (-/-) as compared to wild-type mice (+/+) (*P<0.01, n=4 in each group). All samples were corrected for *gapdh* and expressed relative to the calibrator, which was pooled RNA from adult mouse jejunum.

To quantitatively determine the magnitude of mRNA reduction in the absence of Hnf1α, real-time qRT-PCR was conducted on RNA isolated from adult mid-jejunum using optimized primers for both *LPH* and *SI* mRNAs. *LPH* mRNA was reduced 95% in *hnf1α* null mice as compared to wild-type controls (P<0.01, n=4) whereas *SI* mRNA in *hnf1α* null mice was similar to that in wild-type mice (Fig. 1B). In all analyses, *LPH* and *SI* expression was similar between wild-type and *hnf1α*-/+ mice.

Hnf1α intestinal target genes are reduced throughout the length of the small intestine – To define the importance of Hnf1α for *LPH* and *SI* gene expression throughout the length of the intestine, both of which display regulated proximal-to-distal patterns (16, 36), *LPH* and *SI* mRNA abundance was determined throughout the intestine of wild-type and *hnf1α* null mice using RNase protection assays. As shown in Fig. 2, *LPH* mRNA was greatly reduced throughout the length of the mouse small intestine of *hnf1α*-/- mice as compared to *hnf1α*+/+ controls, whereas *SI* mRNA of *hnf1α* null mice was similar to that of wild-type mice in all segments, with the possible exception of the proximal intestine where *SI* mRNA was higher in the *hnf1α* null mice. This pattern was confirmed by real-time qRT-PCR on post-weaning mice (n = 4 in each group), which also revealed a greatly reduced *LPH* mRNA abundance, and a variable increase in *SI* mRNA levels throughout the intestine of *hnf1α* null mice (not shown).

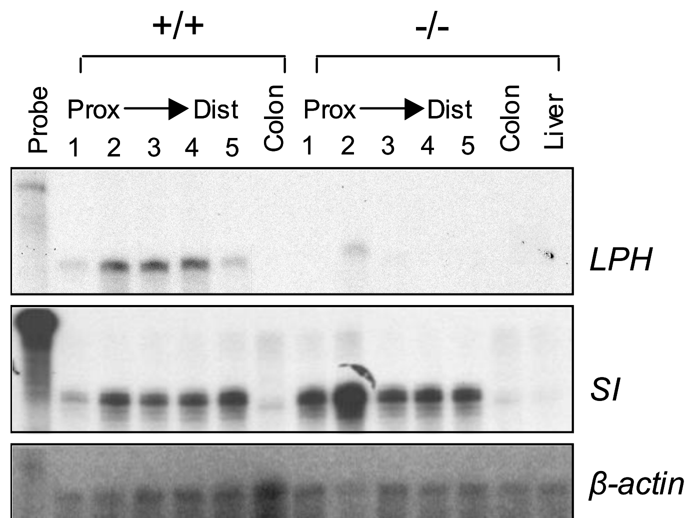

Fig. 2. ***LPH*** **mRNA is reduced throughout the length of the mouse small intestine of adult** ***hnf1α*** **null mice.** RNase protection assays were conducted using probes specific for mouse *LPH*, *SI* and *β-actin* mRNAs and RNA isolated from 5 equidistant segments along the length of the small intestine as described (49), and in distal colon of adult wild-type (+/+) and *hnf1α* null (-/-) mice, as indicated across the top of the figure. Segment 1 corresponds to proximal duodenum; segment 2, proximal jejunum; segment 3, mid jejunum; segment 4, proximal ileum; segment 5, distal ileum. Liver RNA was included as a negative control.

Absence of hnf1α does not affect the expression of other intestinal transcription factors – To ensure specificity of the *hnf1α* null effect on its target genes, the expression of other intestinal transcription factors implicated in the regulation of *LPH* or *SI* was determined in wild-type and *hnf1α-/-* mice (Fig. 3A). As expected, *hnf1α* mRNA was expressed in the adult jejunum of wild-type mice, but was absent in that of the *hnf1α* null mice (Fig. 3A). Absence of Hnf1α was also confirmed by Western analysis and immunohistochemistry (Fig. 3B&C, respectively), as previously documented in the liver of these mice (1). None of the other intestinal transcription

factors expressed in jejunum, including *hnf1β, gata4, gata5, gata6, cdx1 and cdx2,* was affected by the absence of *hnf1α.* However, duodenal *pdx1,* a putative target of Hnf1α (11) and repressor of *LPH* gene expression (53), was reduced in the *hnf1α* null mice. Since duodenal expression of *LPH* is attenuated rather than increased in the *hnf1α* null mice, it is unlikely that decreased *pdx1* affects duodenal *LPH* expression in this model. Noteworthy, is the lack of a compensatory response in *hnf1β* gene expression as shown in the liver of *hnf1α* null mice (41). Together, these data demonstrate that the observed effects on the expression of *LPH* are unlikely due to secondary effects by these transcriptional regulators. However, these findings do not rule out the possibility that as yet unidentified transcriptional regulators of *LPH* are affected by the absence of Hnf1α.

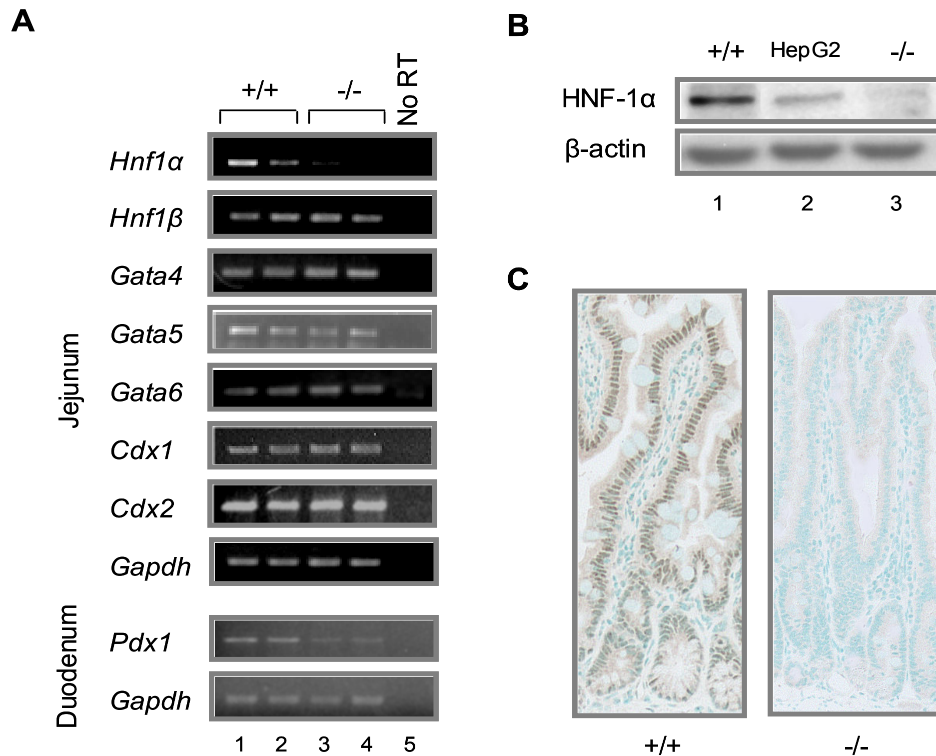

Fig. 3. Intestinal transcription factors are not affected by the absence of Hnf1α. (A) Semiquantitative RT-PCR analysis was carried out using primers specific for *hnf1α, hnf1β*, *gata4*, *gata5, gata6, cdx1*, *cdx2, hnf4α, pdx-1* and *gapdh* with RNA from the jejunum and duodenum of two wild-type (+/+ lanes 1&2) and two *hnf1α* null (-/- lanes3&4) mice. A reaction without reverse transcriptase (No RT) served as a control for DNAcontamination (lane 5). (B) Western analysis for Hnf1α protein was performed as described previously (49). The expression of Hnf1α in wild-type jejunum and HepG2 cells, a hepatocyte-like cell line, was confirmed by the presence of ~90 kD band (lanes1&2) that was absent in the jejunal nuclear extract from an *hnf1α* null mouse (lane 3). As a loading control, the blot was stripped and re-probed with anti-β-actin antibody. (C)Immunostaining with a specific Hnf1α antibody was performed as described previously (49). Nuclei in the epithelial cells of crypts and villi from a wild-type mouse jejunum (+/+) stained positive for Hnf1α, whereas no specific staining was detected in the sections from *hnf1α* null mice (-/-).

Hnf1α binds the mouse LPH and SI promoters both in vitro and in vivo. Although previous reports using EMSAs demonstrated that Hnf1α interacts with specificsites on the *LPH* and *SI* promoters *in vitro* (4, 18), these studies have generally been conducted using rat, pig or human probes and *in vitro* transcribed and translated proteins or proteins derived from cells grown in culture. Thus, to define the association of Hnf1α with the previously identified HNF-1 binding sites on the mouse *LPH* and *SI* promoters under stoichiometric conditions similar to those present in the intestinal epithelium, EMSAs were conducted using mouse probes (mCE-2c & mSIF3) and nuclear extracts obtained from isolated intestinal epithelial cells from adult mouse jejunum (Fig. 4A). For both the *LPH* and *SI* probes, a specific protein-DNA complex was formed, as indicated by specific competition, that completely supershifted when an Hnf1α antibody was added; no detectable supershift complex was formed when an Hnf1β antibody was added. These data indicate that both promoters are capable of binding Hnf1α at physiological levels *in vitro*, and that Hnf1α is the predominant protein in nuclear extracts from intestinal epithelium that binds to these promoters. To test the hypothesis that Hnf1α is associated with the *LPH* and *SI* promoters *in vivo*, ChIP assays were conducted on chromatin isolated from the epithelium of adult mouse jejunum using an Hnf1α antibody. A critical step for valid ChIP assays on chromatin isolated from animal tissue is to establish consistent conditions for chromatin fragmentation prior to immunoprecipitation. Optimal results were achieved when chromatin fragments ranged from 0.1 to 1.0 kb (Fig. 4B). In formaldehyde cross-linked chromatin from *hnf1α*$^{+/+}$ mice, both the *LPH* and *SI* promoters were immunoprecipitated with the Hnf1α antibody (Fig. 4C). These data, which were replicated on three different animals, demonstrate that Hnf1α is associated with the promoters of both the *LPH* and *SI* genes *in vivo*.

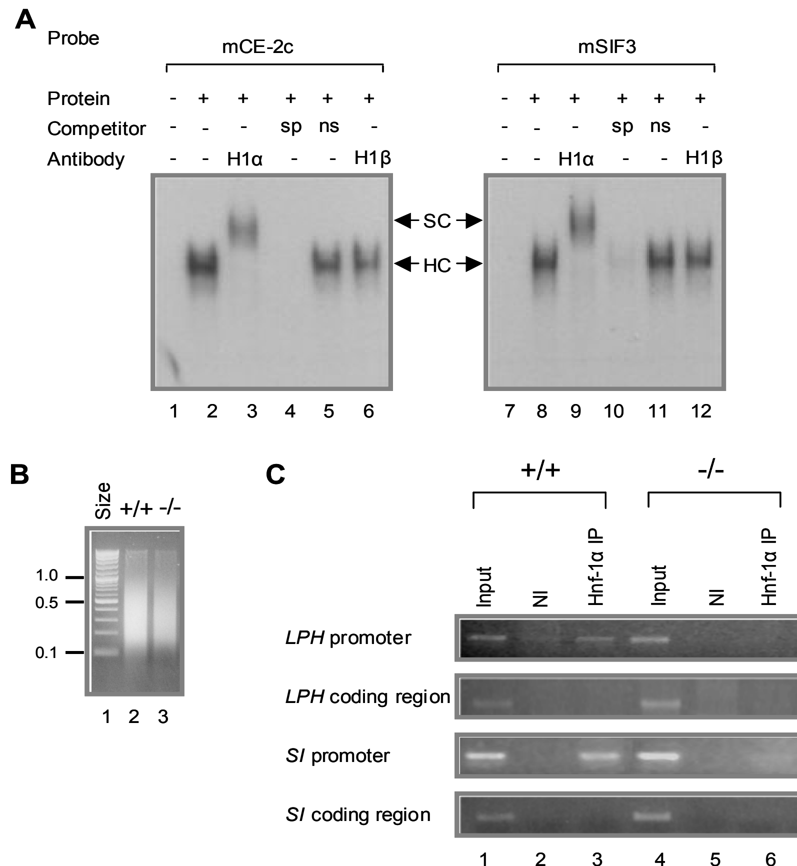

Fig. 4. Hnf1α binds to the *LPH* and *SI* promoters both *in vitro* and *in vivo*. (A) EMSAs were carried out using nuclear extracts (20 μg) isolated from the epithelial cells of the jejunum of adult wild-type mice and a probes containing the HNF-1 binding sites of the mouse *LPH* and *SI* promoters (mCE-2c & mSIF3, respectively). An HNF-1 complex (HC) was formed that supershifted (supershift complex, SC) with the anti Hnf1α antibody (lanes 3 and 9), but not with anti-Hnf1β antibody (lanes 6 and 12). TheHC was competed away by adding an unlabeled specific (sp) oligonucleotide (lanes 4 and10), but was not competed away when a non-specific (ns) competitor was added (lanes 5 and 11). (B) Chromosomal fragmentation was carried out on formaldehyde cross-linked DNA from enterocytes of wild-type (+/+, lane 2) and *hnf1α* null (-/-, lane 3) mouse jejunum by sonication until fragment sizes ranged from 0.1 to 1.0 kb. DNA was separatedin a 1.5% ethidium bromide-stained agarose gel along with a size marker (lane 1). (C) ChIP assays were conducted on fragmented chromatin from wild-type (+/+, lanes 1-3) and *hnf1α* null (-/-, lanes 4-6) mice using an Hnf1α antibody (lanes 3&6). Diluted input DNA samples were assayed in parallel to illustrate that the promoters were present in equimolar amounts in the chromatin of wild-type and *hnf1α* null mice (lane 1&4). Immunoprecipitated samples were analyzed by PCR using primers for the *LPH* or *SI* promoter. Immunoprecipitations using non-immune sereum (NI, lanes 2&5) and amplification of the *LPH* and *SI* coding regions were used as negative controls.

The histone acetylation state at the LPH and SI promoters is not regulated by Hnf1α - In liver and pancreas, Hnf1α has been shown to be indispensable for the recruitment of HAT activity to the promoters of target genes in these tissues. To test the hypothesis that Hnf1α is also

necessary for histone acetylation and subsequent activation of intestinal target genes, ChIP assays were conducted on the *LPH* and *SI* promoters using antibodies specific for acetylated tails of histone 3 and histone 4 (Fig. 5). In wild-type mice, the histones at both the *LPH* and *SI* promoters were highly acetylated which is consistent with an activated or poised state. However, in the absence of Hnf1α, histone 3 and histone 4 remained highly acetylated at both promoters. These data were confirmed in two other pairs of wild-type and *hnf1α* null mice. Although these data are consistent with the hypothesis that Hnf1α is dispensable for the acetylation of histones at the *LPH* promoter, they do not rule out the possibility that subtle changes in histone acetylation occur that are below the limits of detection of this assay, or that redundant or compensatory mechanisms take over in the absence of Hnf1α.

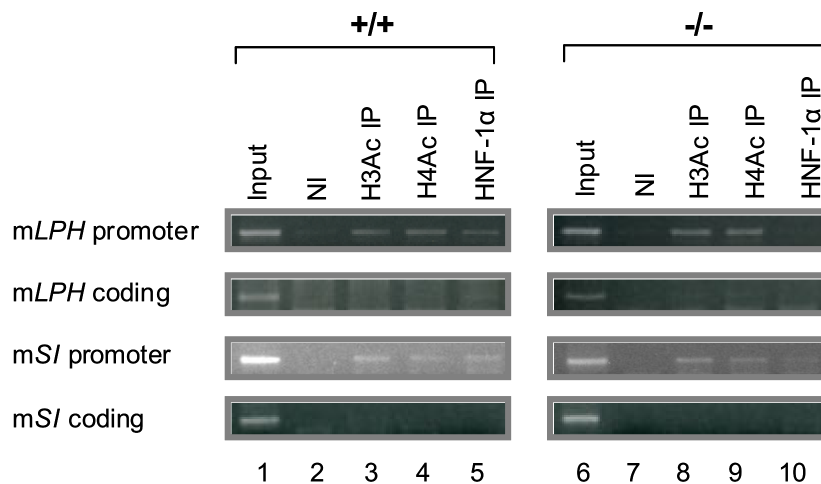

Fig. 5. Nucleosomes at the *LPH* and *SI* promoters remain hyperacetylated in the absence of Hnf1α ChIP assays were conducted on fragmented chromatin from wild-type (+/+, lanes 1-5) and *hnf1α* null (-/-, lanes 6-10) mice using anti-acetylated histone H3 (AcH3, lanes 3&8) and anti-acetylated histone H4 (AcH4, lanes 4&9) antibodies. Diluted input DNA (lanes 1&6) and immunoprecipitations using non-immune serum (NI, lanes 2&7) are also shown. ChIP assays using an Hnf1α antibody (lanes 5&10) were used as a validated control (Fig. 4C).

Hnf1α regulates a subset of intestinal genes in vivo – The differential effect on *LPH* and *SI* gene expression in the *hnf1α* null mice has led us to investigate other genes expressed in the intestine, including those hypothesized to be regulated by Hnf1α in cell cultures studies, and/or those expressed in the intestine, but shown to be regulated by Hnf1α in non-intestinal tissues *in vivo* (Fig. 6). In addition to *LPH* and *SI*, these include *Fabp1*(1, 9), *α1-antitrypsin* (15), *aldolase B* (48), *guanylin* (14), sodium-glucose cotransporter 1(*SGLT1*) (23), *hnf4α* (12), *L-PK* (41) and *neuroD1* (41). Surprisingly, of 8 putative Hnf1α target genes indicated by cell culture assays, only *LPH*, *Fabp1*, α1-*antitrypsin* and *guanylin* mRNAs were reduced (Fig. 6) in the absence of

Hnf1α in adult mouse jejunum; expression of the other putative target genes was indistinguishable from that in wild-type mice. Of the 5 target genes whose expression is reduced in the absence of Hnf1α in adult pancreas or liver, the mRNAs of two (*Fabp1* and α1-*antitrypsin*) were also reduced in the intestine revealing a tissue specificity associated with Hnf1α regulation. Noteworthy, *SGLT1* expression was modestly increased in adult jejunum of *hnf1α* null mice, which we believe is a response to the diabetic phenotype (see Discussion). Since many of the putative Hnf1α targets are developmentally regulated, the importance of Hnf1α in newborn mice was also determined (Fig. 6). *LPH*, *Fabp1*, α1- *antitrypsin* and *guanylin* were all reduced in the absence of Hnf1α in both the newborn and adult small intestine. However, although α1-*antitrypsin* in newborn *hnf1α*-/- mice was reduced similarly to that in adults, *LPH* and *Fabp1* were reduced modestly in newborn *hnf1α*-/- mice, but almost completely in adult *hnf1α*-/- mice. *Guanylin*, on the other hand, was greatly reduced in newborn *hnf1α*-/- mice, but only modestly in adults. These data suggest that Hnf1α is required for the expression of a particular subset of intestinal target genes, but in a specific developmental context.

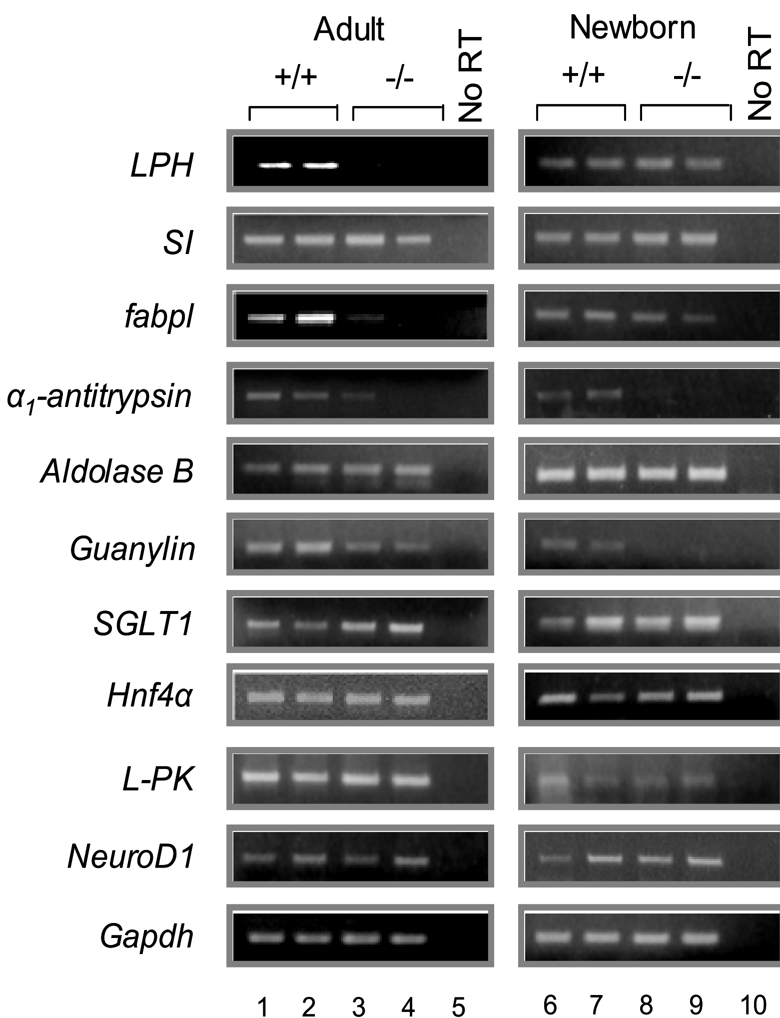

Fig. 6. Hnf1α is required for the expression of a subset of intestinal genes. Semiquantitative RT-PCR was conducted on other genes expressed in the intestine, including those hypothesized to be regulated by Hnf1α in cell cultures studies, and/or those shown to be regulated by HNF-1α in non-intestinal tissues using *hnf1α* null mice (indicated along the left side of the figure). RT-PCR was conducted on RNA isolated from two adult and two newborn wild-type (+/+) and *hnf1*α null (-/-) mice, as indicated across the top of the figure. All targets were optimized to be in the linear range. Reactions without reverse transcriptase (No RT) served as a control for DNA contamination.

DISCUSSION

Establishment and maintenance of intestinal function requires a complex interplay of multiple transcription factors that together coordinate intestinal gene expression. Hnf1α is a transcriptional regulator that has been implicated as an activator of intestinal genes, but whose function in the intestine *in vivo* is only beginning to be elucidated (27, 37, 40, 52). In the present study, we defined the importance of Hnf1α for the expression of intestinal genes using mice that do not express *hnf1α* rather than by relying on *in vitro* and cell culture models. We found that *LPH* gene expression was markedly reduced as anticipated, whereas *SI* gene expression was surprisingly unaffected by the absence of Hnf1α. Despite the differential requirement of Hnf1α for *LPH* and *SI* gene expression, both the *LPH* and *SI* promoters bind Hnf1α in the *in vivo* context of enterocyte chromatin. Analysis of the histone acetylation state at the *LPH* and *SI* promoters revealed that, in contrast to Hnf1α target genes in liver and pancreas where Hnf1α is for recruitment of HAT activity and subsequent transcriptional activation, histone acetylation was not affected by the presence or absence of Hnf1α. Finally, we show that a subset of hypothesized intestinal target genes is regulated by Hnf1α, and this regulation occurs in a defined tissue-specific and developmental context.

Due to compelling *in vitro* and cell culture data demonstrating that Hnf1α binds and activates the *LPH* and *SI* promoters, it was hypothesized that Hnf1α regulates *LPH* and *SI* gene expression *in vivo*. While a significantly reduced expression of LPH in the absence of Hnf1α was anticipated, it was unexpected that *SI* mRNA was unaffected by the absence of Hnf1α in the small intestines of either adult or newborn *hnf1α* null mice. An analyses of others genes previously shown to be targets of Hnf1α using *in vitro* and cell culture assays revealed that some are *in vivo* targets of Hnf1α, includin*, α1-antitrypsin*, and *guanylin,* whereas others are not regulated by Hnf1α *in vivo*, including *SGLT1*, *aldolase B*, *hnf4α,* and *L-PK*. Thus, these data indicate that cell culture studies, while valuable to define putative importance and underlying mechanisms of transcriptional activation, should be interpreted in the context of the defined model, and may not always predict *in vivo* function.

In transgenic mice expressing a reporter under the control of the *SI* or *Fabp1* promoters, mutations in well characterized HNF-1 binding sites reveal a strong reduction in promoter activation in comparison to mice carrying a wild-type transgene (6, 10), demonstrating that these sites are necessary for promoter activation *in vivo*. Since Hnf1α was the predominant protein from intestinal nuclear extracts that binds to these sites (Fig. 4) (4), it was hypothesized that interaction of Hnf1α with these sites is required for *SI* and *Fabp1* gene expression *in vivo* (4, 10). Our data showing that *Fabp1* is a transcriptional target of Hnf1α *in vivo* (Fig. 6) supports this hypothesis for *Fabp1* gene expression, but the lack of a requirement of Hnf1α for *SI* gene expression (Fig. 1&2) is inconsistent with this hypothesis. It is possible that the SI promoter used in these transgenes does not contain all of the HNF-1 sites necessary for activation. It is also possible that the mutations in SIF3 interrupt the binding of another critical activator, such as

Hnf1β. Although the ability of Hnf1β to activate *SI* in cell culture models is minimal (6, 18), it binds to SIF3 *in vitro* (6) and is thus a candidate activator of SI gene expression *in vivo*. It is also possible that an as yet unknown protein binds to SIF3. Precedence for alternative binding to well characterized *cis*-acting elements is supported by similar data on the *SI* promoter where CDP-Cux, a transcriptional repressor of *SI in vivo* (5), binds to sequence that overlaps a conserved GATA binding site in the 5'-flanking region. In conclusion, *SI* is not a transcriptional target of Hnf1α, but its well characterized HNF-1 binding site, SIF3, may be required to mediate *SI* activation *in vivo*.

It can be argued that the effects we observed in the intestine of the *hnf1α* null mice are secondary to the diabetic phenotype. It has been well established in numerous studies in diabetic humans and rodent models that enzymes and transporters involved in intestinal glucose metabolism are increased (26, 28, 45, 56), which probably is a secondary response to hyperphagia, hypoinsulinemia or sensing of glucosuria. Consistent with these studies, we detected a modest increase in the mRNA abundance of SI (Fig. 3 and data not shown), and SGLT1 (Fig. 6) of adult *hnf1α* null mice. However, *LPH* mRNA levels were significantly reduced, and we therefore conclude that the reduced expression of *LPH* in adult *hnf1α* null mice is a direct effect of the lack of Hnf1α, rather than secondary to diabetes.

In ChIP assays, we found in adult wild-type mice that Hnf1α was associated with both the *LPH* and *SI* promoters *in vivo*, yet only *LPH* is regulated by Hnf1α. This discordant relationship between *in vivo* Hnf1α binding to target promoters and Hnf1α gene activation was also found for genes expressed in liver and pancreas. For example, Hnf1α is associated with the promoters of *pah*, *glut2* and *L-PK* in liver and pancreas where these genes are all expressed. However, Hnf1α is only required for *pah* expression in liver, and *glut2* and *L-PK* expression in pancreas. These and our data therefore reveal that association of Hnf1α with a putative target promoter does not necessarily predict regulation by Hnf1α.

Our data reveal that in the absence of Hnf1α, the *LPH* promoter remains hyperacetylated (Fig. 5), and thus the 95% reduction in *LPH* gene expression in adult intestine is not a result of local hypoacetylation of the promoter. This finding contrasts with that in liver and pancreas, where Hnf1α dependency was always associated with histone hyperacetylation of the promoters. For example, in the presence of Hnf1α, such as in wild-type mice, the *pah* gene was expressed in liver where the histones at the promoter were highly acetylated, and the DNA in this region was hypomethylated indicating an active or poised state (29). However, in *hnf1α* null mice, *pah* gene expression was silenced, and the histones and DNA at the promoter were hypoacetylated and hypermethylated, respectively (29, 32). The *glut2* and *L-PK* genes are endogenously expressed in multiple tissues, but in the absence of Hnf1α, *glut2* and *L-PK* gene expression is silenced only in the pancreas. The histones at the *glut2* and *L-PK* promoters, which are normally hyperacetylated in pancreas and liver, were hypoacetylated only in the pancreas of *hnf1α* null mice (29). From these studies, a model was proposed in which Hnf1α promotes and maintains the formation of a transcriptionally competent state through local recruitment of HAT activity (51). Our data show

that the acetylation state of the *LPH* promoter is not different in the epithelium of adult *hnf1α* null mice as compared to controls (Fig. 6). Although it is possible that modest alterations in histone acetylation are below the detection limits of the assay, or that Hnf1α plays a role in histone acetylation, but in its absence, other factors compensate, our data for *LPH* are fundamentally consistent with the hypothesis that Hnf1α is dispensable for the recruitment of HAT activity. These data therefore indicate that other factors are responsible for chromatin modification of the *LPH* promoter.

Our data show that Hnf1α regulates target genes in defined tissue-specific and developmental contexts. For example, *hnf4α, L-PK*, and *neuroD1* are all targets of Hnf1α in non-intestinal tissue *in vivo*, but are not regulated by Hnf1α in the intestine. Further, *LPH*, *Fabp1* and *guanylin* reveal a developmental-specific reduction in gene expression in the absence of Hnf1α in adult intestine. Differential tissue regulation by Hnf1α has been previously shown for target enes expressed in both liver and pancreas (29, 41). Together, these data argue that Hnf1α controls the tissue- and developmental specific expression of target genes through a complex mechanism, likely involving other transcriptional regulators.

The data presented here and in the literature (33) support a role for Hnf1α as a regulator of terminal metabolic genes in different tissues. The intestinal genes shown to be regulated by Hnf1α in the present study have diverse functions, including terminal digestion of nutrients (LPH), intracellular lipid transport (FABP-1), inflammatory response(α1-antitrypsin), and fluid and electrolyte balance (guanylin). Thus, disruption in intestinal Hnf1α regulatory pathways could alter important metabolic processes within the intestine, though this was not specifically studied in the present investigation. Specific mutations in Hnf1α in humans are associated with MODY3 (33), and some of these mutations result in a disrupted interaction with other transcription factors causing a decreased activation of intestinal target genes, such as *Fabp1* (9). Thus, it is possible that individuals with Hnf1α mutations that cause MODY3 could have metabolic consequences in the small intestine.

Hnf1α selectively regulates specific genes in diverse tissues and cell types by a mechanism that is dependent on information in the promoters of these target genes, and the panel of transcriptional regulators present in the tissues or cell types that express these genes. Many intestinal gene promoters have conserved binding sites not only for HNF-1, but also for the Cdx and GATA families of transcription factors (46). We have previously shown that Hnf1α physically interacts with GATA factors, namely Gata4, to cooperatively activate specific promoters, including that of *LPH* and *Fabp1* (10, 18, 49, 50) through an evolutionarily conserved mechanism (49, 50). We have hypothesized that the overlapping expression and subsequent interaction of Hnf1α and Gata4 in the intestinal epithelium is a means to achieve high levels of intestine-specific gene expression of specific targets (49, 50). Hnf1α expression is constant throughout the length of the adult mouse small intestine (49), whereas Gata4 expression declines in distal intestine coincident with a distal decline in *LPH* gene expression. Thus, we propose that although Hnf1α is necessary for *LPH* gene expression in the adult mouse small intestine, its

specific pattern is defined by co-regulation with Gata4. Like *LPH*, the *Fabp1* promoter contains conserved HNF-1 and GATA binding sites (10), and is regulated *in vivo* by both Hnf1α (Fig. 6) and Gata4 (10), further supporting a mechanism of cooperativity between these two transcription factors. These studies indicate that Hnf1α is necessary for the expression of a subset of intestinal genes, and likely interacts with other transcriptional regulators such as Gata4 to mediate the specific pattern of target gene expression. The specific pattern of expression is therefore dependent on a dynamic relationship between the target gene promoter and the transcriptional regulators present within a specific spatial and developmental context.

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CHAPTER 4

Gata4 is essential for the maintenance of jejunal-ileal identities in the adult mouse small intestine

Tjalling Bosse[1], Christina M. Piaseckyj[2], Ellen Burghard[1], John J. Fialkovich[2], Satish Rajagopal[3], William T. Pu[3], Stephen D. Krasinski[2,4]

From the[1]Department of Pediatrics, University of Amsterdam, Amsterdam, The Netherlands 1100DD; and Departments of [2]Medicine and [3]Cardiology, Childrens Hospital and Harvard Medical School, Boston, Massachusetts, 02115; and [4]Dorothy R. Friedman School of Nutrition Science and Policy, Tufts University, Boston, Massachusetts, 02111

ABSTRACT

Gata4, a member of the zinc finger family of GATA transcription factors, is highly expressed in duodenum and jejunum, but is nearly undetectable in distal ileum of adult mice. We show here that the caudal reduction of Gata4 is conserved in humans. To test the hypothesis that the regional expression of Gata4 is critical for the maintenance of jejunal-ileal homeostasis in the adult small intestine in vivo, we established an inducible, intestine-specific model that results in the synthesis of a transcriptionally inactive Gata4 mutant. Synthesis of mutant Gata4 in jejunum of 6-8 week old mice resulted in an attenuation of absorptive enterocyte genes normally expressed in jejunum but not in ileum, including the anticipated targets liver fatty acid binding protein (*Fabp1*) and lactase-phlorizin hydrolase (*LPH*), and a surprising induction of genes normally silent in jejunum but highly expressed in ileum, specifically those involved in bile acid metabolism. In the jejunum of *Gata4* mutant mice, goblet cell population was increased ~40% ($P<0.05$), and mRNAs for specific enteroendocrine subpopulations were redistributed toward an ileal phenotype. *Math1*, a known activator of the secretory cell fate, was induced ~75% ($P<0.05$). Gata4 is thus an important positional signal required for the maintenance of jejunal-ileal identities in the adult mouse 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 (13). The functional diversity is linked to a continuous renewal process in which stem cells located 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, goblet cells, and enteroendocrine cells, migrate up the villi and are shed into the intestinal lumen every 3-5 days, whereas Paneth cells reside at the base of crypts and turn over more slowly. Absorptive enterocyte genes that encode proteins responsible for the terminal digestion and absorption of most nutrients are expressed primarily in duodenum and jejunum, whereas those that mediate absorption of conjugated bile salts and intrinsic factor-bound vitamin B_{12} are localized to the distal ileum. Goblet and Paneth cells are more numerous in distal small intestine presumably to provide greater protection against bacterial infiltration, and enteroendocrine subpopulations display a functional diversity characterized by the regional segregation of hormones that activate or repress gastrointestinal processes. Although gene knockout and over-expression models have resulted in the discovery that Wnt, Hedgehog, and Notch signaling are critical regulatory pathways for intestinal differentiation in vivo (1), the embryonic lethality of many of these models has hampered our ability to understand the processes involved in the maintenance of regional identities in the mature intestine.

Gata4 is a member of an evolutionarily conserved family of zinc-finger transcription factors necessary for a variety of developmental processes including embryonic morphogenesis and cellular differentiation (24). Inactivation of GATA homologs in lower animals, such as *elt-2* in *C. elegans* (11), *serpent* in *D. melanogaster* (29), or *gata4* in zebrafish (15), result in defects in heart development and gastrointestinal organogenesis demonstrating a conservation of function. In mice, Gata4 is expressed in visceral endoderm in embryos, and in developing and mature heart, ovary, testis, pancreas, liver, stomach, and small intestine. *Gata4* inactivation models in mice reveal abnormalities in ventral morphogenesis resulting in a failure to form a primitive heart tube and foregut (20, 25), as well as specific defects in cardiac morphogenesis and proliferation later in embryonic development (28, 45, 47). However, due to the embryonic lethality of these models, the importance of Gata4 for intestinal function in vivo in mature mice is currently unknown.

Gata4 is highly expressed in duodenum and jejunum, but is greatly reduced in distal ileum of adult mice (43) implying a regulatory role along the cephalo-caudal axis. It is not known if this regional pattern occurs in humans. Gata4 is expressed in absorptive enterocytes on villi (3, 7, 9, 43) where it is thought to trans-activate specific target genes (3, 7, 8, 10, 12, 26, 43). Isolated *Gata4*-/- cells in the small intestine of chimeric E18.5 mice do not express the intestinal differentiation marker, liver fatty acid binding protein (*Fabp1)* (7), providing key in vivo data demonstrating a critical importance for Gata4 in the regulation of intestinal gene expression, and

a lack of overlapping functions with other intestinal GATA factors. We have shown that Gata4 binds and activates in vitro the promoter of the putative absorptive enterocyte target, lactase-phlorizin hydrolase (*LPH*). We have further shown that Gata4 physically associates with hepatocyte nuclear factor-1α (Hnf1α), a homeodomain transcription factor also expressed in the intestinal epithelium (2, 3, 33), and synergistically activates the LPH promoter through an evolutionarily conserved mechanism (43, 44). Finally, we have hypothesized that the known distal decline in *LPH* gene expression is linked to the caudal reduction in Gata4 expression (43). In the present study, we established an inducible, intestine-specific *Gata4* knockout model to test the hypothesis that Gata4 is required for the maintenance of jejunal-ileal patterns of gene expression in the adult small intestine in vivo.

MATERIALS AND METHODS

Mice. Mice were housed under standard conditions in the Animal Research at Children's Hospital (ARCH) facility and provided food and water *ad libitum*. *Gata4*$^{flox/flox}$ mice (28) were crossed with transgenic Villin-*Cre*ER^{T2} mice (21) (generous gift of Dr. S. Robine, Institut Curie, Paris) to generate experimental *Gata4*$^{flox/flox}$, Villin-*Cre*ER^{T2} mice. In these mice, *Cre* is expressed in the epithelial cells of the small and large intestine. The expressed *Cre* is fused to a mutated estrogen receptor, and resides in the cytoplasm until it is translocated into the nucleus upon Tamoxifen™ treatment, where it then excises floxed DNA. *Gata4*$^{flox/flox}$ mice positive for the Villin-*Cre*ER^{T2} transgene were designated as *Gata4* mutant mice, whereas *Gata4*$^{flox/flox}$ / Villin-*Cre*ER^{T2}-negative littermates were used as controls.

Genotyping was conducted on tail DNA as described (2) using specific primers for *Cre* and exon 2 of *Gata4*. Nuclear translocation of *Cre* was induced in mice at 6-8 weeks of age by five daily intraperitoneal injections of Tamoxifen™ (100µl, 10mg/ml) as described (14, 21), and study mice were sacrificed 14 days after the last injection. During this 14-day span, mice were weighed, and were observed daily for general activity, skin and hair outlook, and stool firmness. At the time of sacrifice, mice were anesthetized with avertin anesthesia, and the intestine was removed from a midline incision, placed on a glass plate on a bed of ice, and rinsed with ice-cold PBS. Intestinal tissue was obtained along the length of the small intestine for isolation of RNA and nuclear extracts, and sectioning as described (2, 43). Approval was obtained from the Institutional Animal Care and Use Committee for all mouse experiments described here.

Immunofluorescence. Intestinal segments were flushed with ice cold PBS, fixed for 4 h in 4% paraformaldehyde at 4°C, rinsed with 70% ethanol in PBS, and embedded in paraffin. Five µm sections were heated for 15 min at 65°C, deparaffinized and rehydrated. Antigen retrieval was performed by boiling slides for 10 min in 10mM sodium citrate, pH 6.0. After cooling, slides were washed 3X for 5 min in PBS and incubated in a blocking solution containing 10% donkey serum in PBS for 1 h in a humidified chamber at room temperature. Blocking serum was replaced by the primary antibody in 10% donkey serum/PBS, and incubated overnight at

$4^{o}C$ in a humidified chamber. Slides were then washed 3X for 5 min in PBS and incubated with the secondary antibody 10% donkey serum/PBS for 4 h in a humidified chamber at room temperature. In some experiments, a solution containing DAPI (4′,6-diamino-2-phenylindole dihydrochloride) nucleic acid stain (2ug/ml; D1306, Molecular Probes) in PBS was added and incubated for 15 min at room temperature. Slides were washed in PBS, mounted in Mowiol Mounting Media (Calbiochem, San Diego, CA) and allowed to dry overnight.

The primary antibodies included mouse anti-Gata4 (1:250, sc-25310, Santa Cruz), rabbit anti-Gata4 (1:100, sc-9053, Santa Cruz), goat anti-Gata4 (1:500, sc-1237, Santa Cruz), rabbit anti-Gata6 (1:20, sc-9055, Santa Cruz), goat anti-Gata5 (1:40, 1:400, sc-7280, Santa Cruz), rabbit anti-Gata5 (1:40, 1:400, sc-9054), rabbit anti-chromogranin A (1:1000, 20085, Immunostar), rabbit anti-lysozyme (1:200, 18-0039, Zymed), rabbit anti-Ki67 (1:100, RM-9106, Lab Vision), rabbit anti-Fabp1 (1:1000, kind gift of Dr. J. Gordon, Washington University), rabbit anti-Asbt (1:500, kind gift of Dr. P. Dawson, Wake Forest University), and anti-cleaved caspase-3 (1:100, 9664, Cell Signaling Technology). The secondary antibodies used were Alexa fluor 594 donkey anti-goat antibody, Alexa fluor 488 donkey anti-mouse antibody, and Alexa fluor 488 donkey anti-rabbit antibody (1:500, Molecular Probes).

Semi-quantitative and real-time RT-PCR. Semi-quantitative and real-time RT-PCR was conducted as previously described (2). Briefly, RNA was isolated using the RNeasy kit (Qiagen) with DNase treatment (Ambion), quantified by optical density at A260nm, and checked on an agarose gel for intact ribosomal RNA bands. Complementary DNA (cDNA) was synthesized using iScript (BioRad) for semi-quantitative RT-PCR, and intrinsically using the one-step method for real-time RT-PCR. Primer pairs were designed using Beacon Design software (Biosoft International) and optimized as described (2). 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 Green Supermix (Bio-Rad) on 4-6 animals in each group, corrected for *Gapdh* and expressed relative to the calibrator, which was either adult jejunal or adult ileal RNA. RT-PCR primer sequences are available upon request.

Western Blot. Western blot analysis was performed as previously described (43) using 25µg of nuclear extracts from the jejunum of control and *Gata4* mutant mice, and an affinity-purified goat polyclonal antibody directed against the C-terminal domain of Gata4 (sc-1237, Santa Cruz).

Electrophoretic mobility shift assays (EMSAs). EMSAs were carried out as previously described (43) using nuclear extracts from isolated epithelial cells and a probe that specifically binds GATA proteins. For supershift analyses, a goat polyclonal Gata4 antibody (0.1 µg/µl, sc-1237X, Santa Cruz Biotechnology,) was pre-incubated with the nuclear extracts for 20 min prior to the addition of the probe.

Cloning and construction of a mutant Gata4 expression vector. During the course of these studies, we found that the mutant mice produced a truncated form of Gata4, termed $Gata4^{\Delta ex2}$, in the intestine. To clone $Gata4^{\Delta ex2}$ for further analysis, total RNA was isolated from

$Gata4^{wt/\Delta ex2}$ heart tissue, and reverse transcribed using random hexamers and Superscript II (Invitrogen). Complementary DNA was PCR-amplified using primers spanning exon 2, and a 450bp amplicon containing the mutant 5′ end was subcloned into the pCRII-TOPO plasmid (Invitrogen). The mutant 5′ end was then excised by digestion of internal HindIII and MfeI restriction sites, and used to replace the 5′ end of the full-length, wild-type *Gata4* cDNA. HindIII/NotI digests were used to transfer the 1.3kb mutant *Gata4* cDNA into the pcDNA3.1(+) expression vector.

Transient co-transfection. Transient co-transfection assays were carried out in HeLa cells using expression vectors for wild-type or mutant Gata4, and Gata6, and a 118 bp mouse LPH promoter-human growth hormone reporter construct (hLPH-hGH) as described (43). Expression of the promoter-reporter construct by wild-type or mutant Gata4 was expressed relative to that obtained from the empty expression vector, pRC-CMV. Experiments were repeated three times in triplicate, and statistically significant differences were determined by the Student's *t*-test. HeLa cells were tested before and after experimentation to document an absence of mycoplasma infection using the MycoTect™ kit (Invitrogen).

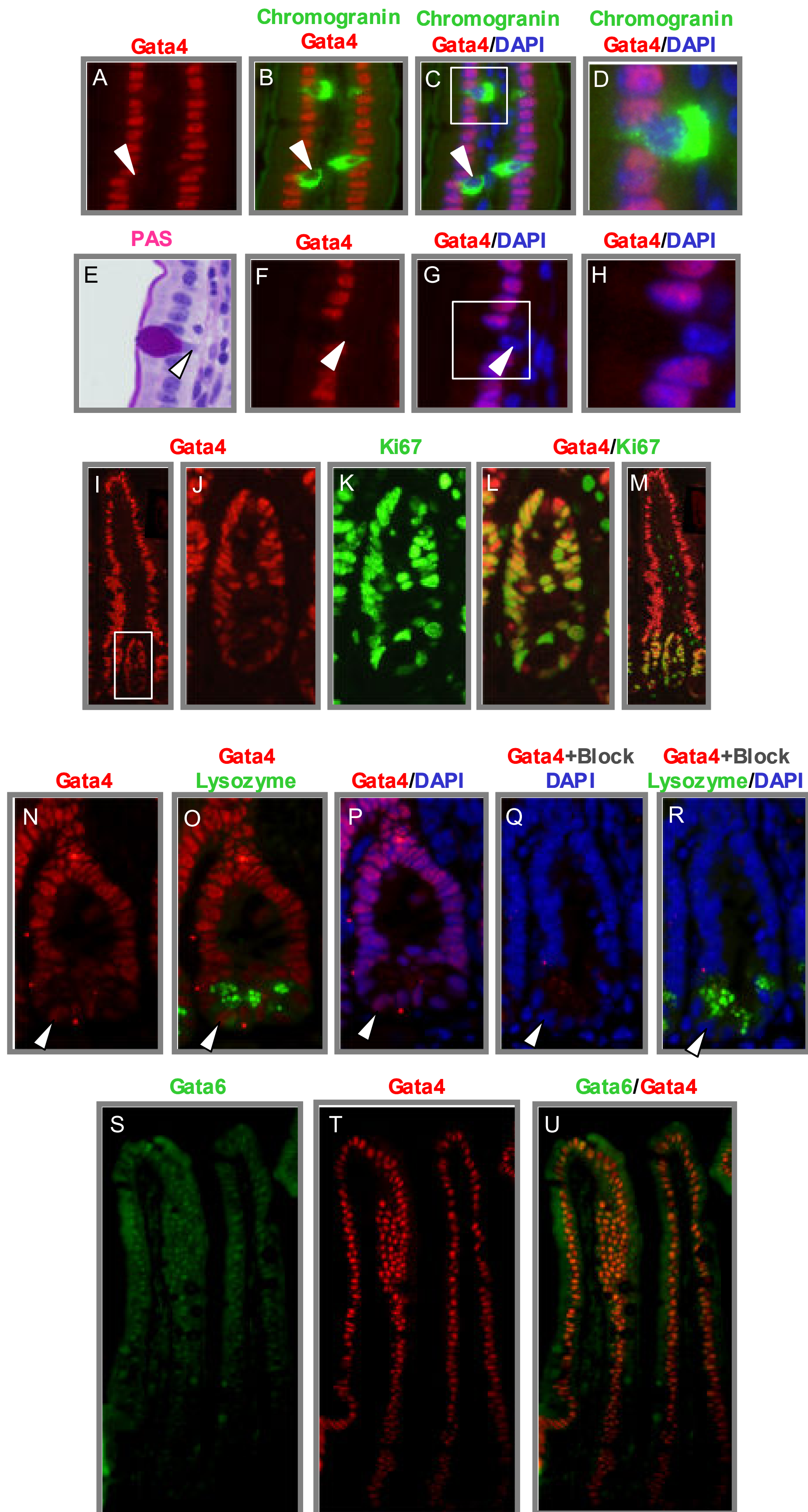
Statistical analyses. Statistically significant differences were determined by the Student's t-test or analysis of variance followed by the Tukey-Kramer multiple comparison test. Goblet cell counts were performed by 8 independent, masked observers counting a total of 10 villi on PAS-stained slides. Data were expressed as the mean of 6 control and 6 *Gata4* mutant mice.

RESULTS

Gata4 is expressed in absorptive enterocytes on villi and in crypt epithelium. Although it is well recognized that Gata4 is highly expressed in absorptive enterocytes on villi (3, 7, 9, 43), its expression in proliferating crypt cells and its co-expression with other Gata factors has not been fully appreciated. We compared three different anti-Gata4 antibodies by immunofluorescence, and found that goat anti-Gata4 produced immunostaining with the highest sensitivity and lowest background. Using this antibody, and an antibody for the enteroendocrine marker, chromogranin A (Fig. 1A-D), we found no evidence of Gata4 in this lineage, as previously shown (9). Using serial sections that included PAS-stained slides, and a criterion that goblet cell nuclei must be outside of the plane of absorptive enterocyte nuclei, we also found no evidence of Gata4 in this lineage (Fig. 1E-H), also as previously shown (9). Gata4 immunofluorescence, however, was found throughout the crypt epithelium (Fig. 1I and J), though at a lower intensity than that which occurs on villi. Gata4 was co-expressed with the marker for proliferating cells, Ki-67, in the nuclei of epithelial cells of the upper crypt (Fig. 1J-M), and lysozyme-positive Paneth cells at the base of the crypt (Fig. 1N-P). Blocking experiments using the specific peptide that produced the goat anti-Gata4 antibody demonstrated that the crypt staining was specific for the Gata4 antibody. Although background immunofluorescence in secretory granules of Paneth cells

remained detectable (Fig. 1Q), the blocking peptide completely attenuated Gata4 immunostaining in crypt nuclei (Fig. 1Q and R), demonstrating that the Gata4 immunostaining in crypts was not due to non-specific immunofluorescence. Gata6 was co-expressed with Gata4 in absorptive enterocytes on villi (Fig. 1S-U), in agreement with previous studies (7, 37), but the presence of Gata6 in either enteroendocrine cells, as previously reported (9), or in crypts (not shown), could not be documented. Although Gata5 mRNA is expressed in mouse small intestine (8), we could not demonstrate Gata5 protein using two different commercial antibodies, despite positive staining in stomach with one of these antibodies (not shown). Thus, on villi, Gata4 is localized specifically to the absorptive enterocyte lineage where it is co-expressed with Gata6. In crypts, Gata4 is expressed throughout the epithelium, including proliferating Ki-67-positive cells and non-proliferating Paneth cells.

Fig. 1. Gata4 is co-expressed with Gata6 in absorptive enterocytes on villi, and throughout the crypt epithelium in the adult mouse jejunum. (A-D) Immunofluorescence showing that Gata4 (red) is not expressed in the nuclei (DAPI, blue) of chromogranin A (green) enteroendocrine cells. Arrowheads indicate the absence of Gata4 in the nucleus of a specific enteroendocrine cell. (E-H) Serial section of a PAS-stained goblet cell showing absence of Gata4 immunofluorescence (red) in the nucleus (DAPI, blue) of this cell (arrowhead). (I-M) Co-immunofluorescence for Gata4 (red) and Ki67 (green) showing that Gata4 is expressed in proliferating epithelial cells of the upper crypt (yellow). (N-P) Co-immunofluorescence for Gata4 (red) and lysozyme (green) showing that Gata4 is expressed in the nucleus (DAPI, blue) of Paneth cells. (Q,R) Serial section using a blocking peptide showing that the nuclear Gata4 expression is not due to non-specific fluorescence. (S-U) Co-immunofluorescence for Gata6 (green) and Gata4 (red) showing that these Gata factors are co-expressed in the absorptive enterocytes on villi (yellow).


Gata4
Chromogranin
Gata4
Chromogranin
Gata4/DAPI
Chromogranin
Gata4/DAPI
A
B
C
D
PAS
Gata4
Gata4/DAPI
Gata4/DAPI
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F
G
H
Gata4
Ki67
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Absence of Gata4 in the ileum is conserved in humans. Quantification of *Gata4* mRNA in five equidistant segments along the length of the adult mouse small intestine by real-time RT-PCR revealed that *Gata4* mRNA abundance in the most distal segment (segment 5) was ~5% of that in all other intestinal segments studied ($P<0.05$) demonstrating a sharp decline in *Gata4* expression localized to the distal ileum (Fig. 2A). This is consistent with our previous Western analysis which showed a greatly reduced abundance of Gata4 protein in distal ileum as compared to all other segments of adult mouse small intestine (43). Gata4 was expressed in the epithelial cells of the adult human jejunum (Fig. 2B), but was not detected in human ileum (Fig. 2C), as determined by immunofluorescence. H&E staining (Fig. 2D and E), and positive nuclear staining for Hnf1α (data not shown) confirmed the integrity of the tissue sections. These data demonstrate that the regional expression of *Gata4* is conserved in humans, and that data obtained from mice on the regulation of anterior-posterior homeostasis by Gata4 is likely relevant to humans.

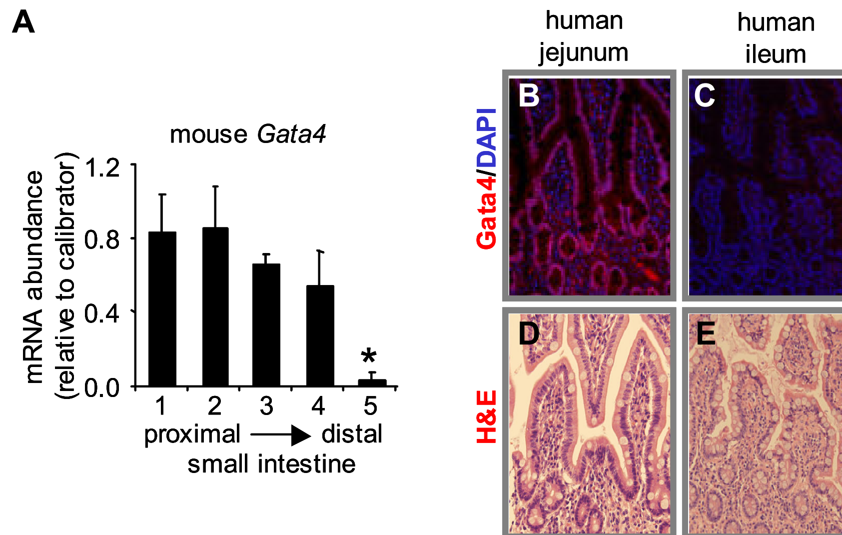


Fig. 2. Absence of Gata4 in ileum is conserved in humans. (A) Real-time RT-PCR analysis of *Gata4* mRNA abundance along the length of the adult mouse small intestine showing that *Gata4* mRNA is significantly lower in the distal ileum as compared to all other segments (*$P<0.05$, mean±SD, n=3). (B,C) Immunofluorescence of adult human intestinal epithelium for Gata4 (red) and nucleic acid by DAPI (blue) showing the presence of Gata4 in jejunum (B), and absence of Gata4 in ileum (C). (D,E). H&E staining of adult human jejunum and ileum showing intact morphology.

Establishment of an inducible, intestine-specific *Gata4* inactivation model. To overcome the limitation of the early lethal phenotype in germline *Gata4* null mice, we established an inducible, intestine-specific *Gata4* inactivation model (Fig. 3). In this model, a previously validated mouse line was used in which a portion of exon 2 containing the translational start site and N-terminal activation domains of Gata4 was flanked by loxP sites (floxed) (Fig. 3A) (28). Germline *Cre*

expression in mice homozygous for the *Gata4* floxed allele (*Gata4$^{flox/flox}$*) exhibited a phenotype that was indistinguishable from that in published germline *Gata4* knockout models (20, 25) (data not shown) verifying these mice. *Gata4$^{flox/flox}$* mice were mated with an established transgenic line (Villin-*Cre*ER^{T2}) in which *Cre* is expressed in the epithelial cells of the small and large intestine under the control of the villin promoter (Fig. 3A) (21). The expressed *Cre* is fused to a mutated estrogen receptor, and resides in the cytoplasm until it is translocated into the nucleus upon Tamoxifen^{TM} treatment, where it then excises floxed DNA. The Villin-*Cre*ER^{T2} transgene is expressed in stem cells since recombination is stably maintained for up to 60 days after Tamoxifen^{TM} treatment (21). As shown in Fig. 3B, *Gata4* was expressed normally in stomach, pancreas, and heart, but was inactivated in the jejunum of *Gata4* mutant mice. Recombination was complete along the length of the small intestine with the exception of the duodenum where exon 2 was not excised completely (Fig. 3B). This was confirmed by experiments using Rosa26 mice (38) which revealed Cre excision specifically in small and large intestine, but not in duodenum (data not shown). Inactivation of Gata4 in the jejunum was complete from 2 days to 4 weeks after the completion of Tamoxifen™ treatment (data not shown).

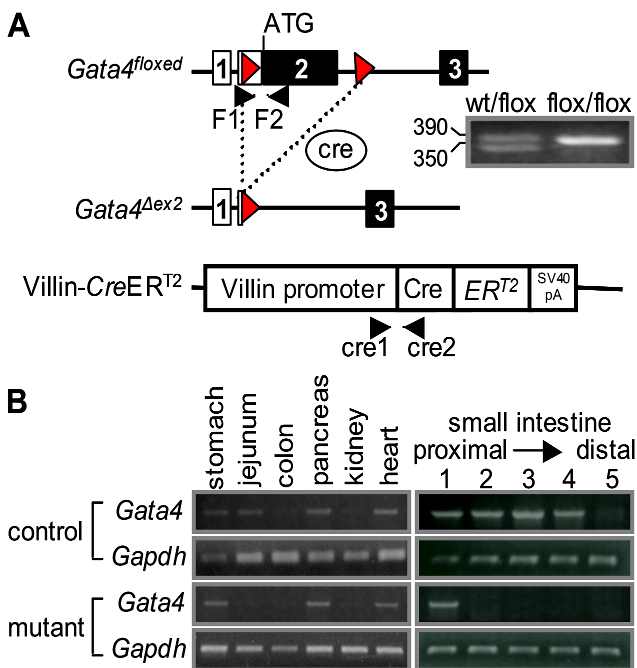


Fig. 3. Villin-*Cre*ER^{T2}-mediated recombination of the *Gata4*^{floxed} allele in mouse small intestine. (A) Schematic representation of the *Gata4* and Villin-*Cre*ER^{T2} alleles used. The *Gata4^{floxed}* allele encodes wild-type Gata4 whereas the *Gata4*$^{\Delta ex2}$ allele represents the *Gata4* locus after Cre-mediated recombination. Arrows indicate location of primers used for genotyping and the inset shows the PCR products that distinguish the wild-type (350bp) from the floxed (390bp) *Gata4* allele. (B) Semi-quantitative RT-PCR analyses of exon 2 using the F1 and F2 primers reveal normal *Gata4* expression in stomach, pancreas, and heart, but null expression in the small intestine of the mutant mice, with the exception of duodenum (segment 1) where *Gata4* mRNA remains detectable.

Western analysis using nuclear extracts isolated from the jejunum of control and *Gata4* mutant mice and an antibody specific for the C-terminal domain of Gata4 revealed that the mutant mice synthesize a truncated form of Gata4 (~33kD, Gata4$^{\Delta ex2}$) in the jejunum (Fig. 4A). Quantitative analysis of nuclear extracts revealed that the abundance of Gata4$^{\Delta ex2}$ in mutant jejunum was similar to that of Gata4 in control jejunum (n=4 in each group, data not shown). Although Gata4$^{\Delta ex2}$ was capable of binding to DNA as shown using EMSAs (Fig. 4B), it was unable to activate the LPH promoter in cell culture co-expression experiments (Fig. 4C),

presumably due to the deletion of its N-terminal activation domains. However, it clearly demonstrated dominant-negative activity in vitro, as shown by its ability to compete the Gata4 or Gata6 activation of the human LPH promoter in transient co-transfection assays (Fig. 4C). The predicted structure (Fig. 4D) is based on excision of the native translational start site and activation domains in exon 2, translation of a predicted in-frame ATG in exon 3, the size of the protein on Western blots (Fig. 4A), and recognition by a C-terminal-directed antibody. These data demonstrate that treatment of *Gata4* mutant mice with Tamoxifen™ results in the synthesis of a truncated, transcriptionally inactive form of Gata4 in the jejunum that has the potential to act as a dominant-negative Gata transcription factor.

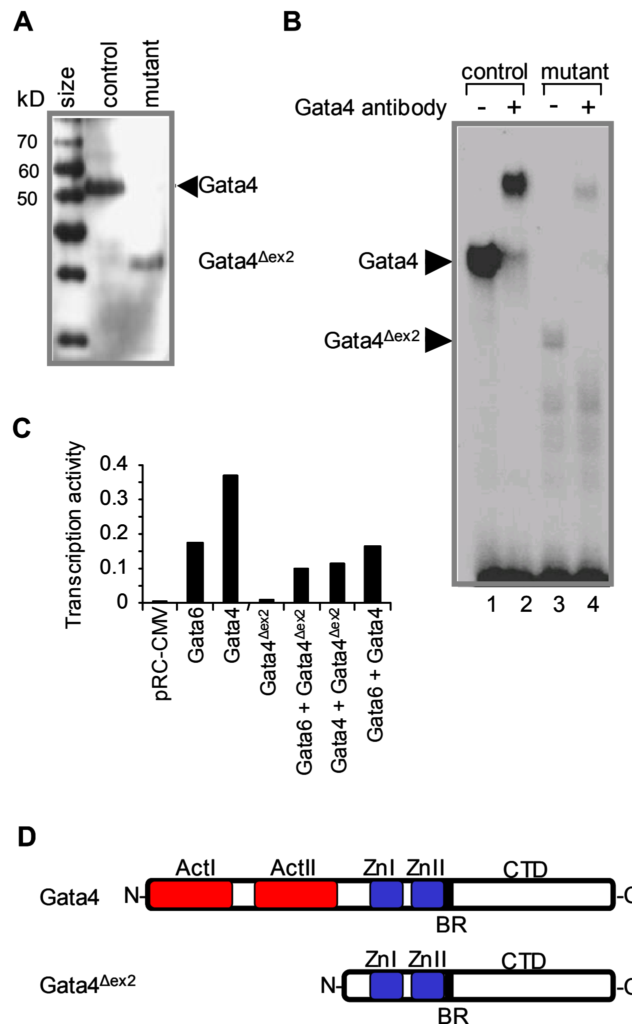


Fig. 4. An inactive, truncated form of Gata4 with dominant-negative activity is synthesized in the jejunum of *Gata4* mutant mice. (A) Western blot analysis using an antibody specific for the C-terminal domain of Gata4 demonstrates a specific band of ~54 kD in the jejunum of control mice (Gata4) and absence of this band in *Gata4* mutant animals. In the *Gata4* mutant mice, another specific band of ~33 kD reveals the presence of a truncated Gata4 protein (Gata4$^{\Delta ex2}$). Identical results were obtained from three other mutant animals. (B) EMSA using a standardized GATA binding site as a probe (43) and nuclear extracts from control and *Gata4* mutant mice showing that both Gata4 and Gata4$^{\Delta ex2}$ bind DNA. Formation of a supershift complex using an antibody directed against the C-terminal domain demonstrates that both Gata4 proteins contain intact C-termini. (C) Transient co-transfection assay in HeLa cells demonstrating that Gata4$^{\Delta ex2}$ is transcriptionally inactive, but has dominant-negative properties. Gata6 and Gata4 individually activated the human LPH promoter/human growth hormone reporter (hLPH-hGH), but Gata4$^{\Delta ex2}$ did not. Gata6 and Gata4 activation of hLPH-hGH was attenuated when Gata4$^{\Delta ex2}$ was co-transfected indicating dominant-negative activity. Co-transfection of Gata6 and Gata4 resulted in an antagonistic response suggesting that Gata6 and Gata4 compete for binding to the hLPH promoter. (D) Schematic representation of the predicted Gata4 protein synthesized in the jejunum of the *Gata4* mutant mice showing the deletion of the N-terminal activation domains (ActI & ActII), but intact zinc fingers (ZnI & ZnII), basic region (BR), and C-terminal domain (CTD).

Absorptive enterocytes in the jejunum acquire an ileal-like gene expression program in the *Gata4* mutant mice. During the two-week interval between the completion of Tamoxifen treatment and the end of the study, it was noted that body weight, general activity, skin and hair outlook, and stool firmness of the Gata4 mutant mice were indistinguishable from controls. Immunostaining for Fabp1, a marker of intestinal differentiation that is highly expressed in jejunum but nearly undetectable in ileum of adult mice (36), and a confirmed Gata4 target both in vitro and in vivo (7), revealed that Fabp1 was expressed normally in the cytoplasm of absorptive enterocytes of control jejunum (Fig. 5A), but was greatly reduced in absorptive enterocytes of *Gata4* mutant jejunum (Fig. 5B), corresponding to levels characteristic in distal ileum of control mice (Fig. 5C). To test whether the inactivation of Gata4 in the jejunum results in the induction of enterocyte genes normally absent in the jejunum but expressed in ileum, the expression of the ileal apical sodium-dependent bile acid transporter (Asbt=SLC10A) was analyzed. In jejunum, Asbt is normally absent (Fig. 5D), but in the jejunum of *Gata4* mutant mice, Asbt was markedly induced (Fig. 5E), approaching the expression in control ileum (Fig. 5F).

Quantitative analysis revealed that jejunal *Fabp1* mRNA in the *Gata4* mutant mice was ~20% ($P<0.05$) of that in control mice (Fig. 5G), confirming the decrease in immunofluorescence signal. Other hypothesized targets of Gata4 include the genes that encode the disaccharidases LPH (43) and sucrase-isomaltase (SI) (3, 19), as well as intestinal fatty acid binding protein (Fabp2) (12). In mice, *LPH* is normally highly expressed in jejunum but is low or undetectable in distal ileum (18), whereas *SI* and *Fabp2* are normally expressed in both jejunum and ileum (22, 41). In *Gata4* mutant mice, *LPH* mRNA abundance was significantly attenuated (~10% of controls, $P<0.05$), whereas the mRNAs for *SI* and *Fabp2* were only marginally reduced by the inactivation of *Gata4* (Fig. 5G).

The unexpected induction in Asbt was confirmed by real-time RT-PCR, which revealed a 20-fold increase ($P<0.01$) in *Asbt* mRNA in the absence of *Gata4* in the jejunum (Fig. 5G). The expression of the ileal lipid binding protein (Ilbp=Fabp6), an ileal absorptive enterocyte marker (6) thought to be important for intracellular bile acid transport, was also markedly induced (644-fold, $P<0.001$) in the jejunum of *Gata4* mutant mice (Fig. 5G) demonstrating that the induction of ileal genes is not exclusive to *Asbt*.

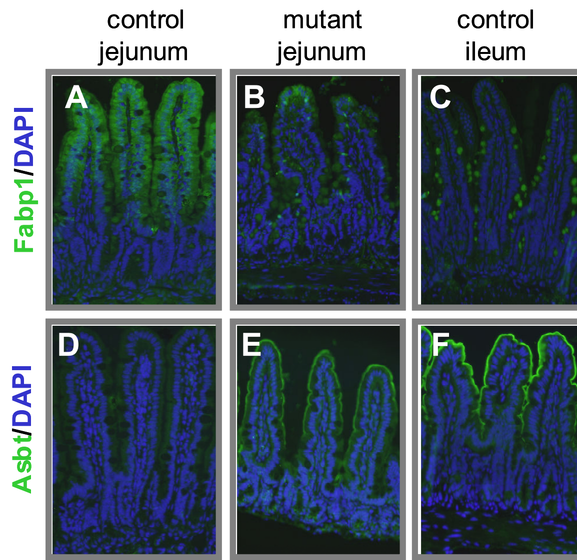
To define the extent of the transformation in jejunum to an ileal-like phenotype, the mRNA abundance of *LPH* and *Asbt* in mutant mice was quantitatively compared to that in control ileum. As shown in Fig. 5H, both *LPH* and *Asbt* mRNA changed in the direction of the ileum, but did not attain ileal levels, indicating that the transformation to an ileal gene expression program in absorptive enterocytes is not complete. It is possible that Gata4 is not completely inactivated, and thus, could account for an incomplete transformation, although our RT-PCR data indicate complete Gata4 inactivation. It is also possible that Gata4 is only partially required for transformation, and that other as yet unknown factors are also involved.

Gata5, Gata6, and Cdx2 have been implicated as transcriptional activators of *Fabp1* and/or *LPH* in vitro (7, 42), and Hnf1α is indispensable for the expression of these two genes in vivo (2). We found no difference in *Gata5*, *Gata6, Cdx2*, or *Hnf1α* mRNA levels between control and *Gata4* mutant mice indicating that the attenuation of *Fabp1* and *LPH* is not secondary to changes in the expression of these regulators (Fig. 5I). In cardiac tissue, Gata6 is up-regulated in the absence of Gata4 (20, 25), but this does not appear to be the case in the small intestine.

The molecular control of *Asbt* gene expression in the mouse ileum has been studied in detail, and Hnf1α (34), the AP-1 proteins c-Jun and c-Fos (4), and liver receptor homologue-1 (Lrh1) (5) have been implicated as activators of *Asbt* gene expression. In the jejunum of *Gata4* mutant mice, the mRNA abundance of *Hnf1α, c-Jun, c-Fos* and *Lrh1* was indistinguishable from that in control jejunum (Fig. 5H) and ileum (data not shown), indicating that the induction of *Asbt* is not secondary to an increase in these activator

Inactivation of *Gata4* results in a jejunal-to-ileal shift in secretory cell composition. Although differential expression of specific absorptive enterocyte genes between jejunum and ileum is readily detectable, differences in composition and gene expression of the secretory lineages (Paneth, goblet, and enteroendocrine cells) between jejunum and ileum are more difficult to quantify due to the subtlety of the regional differences. This is particularly true of Paneth cells, in which we have been unable to document differences in cryptdin1, cryptdin4, and lysozyme mRNA abundance between control jejunum and ileum (data not shown). As a consequence, we found no difference in Paneth-specific expression of these genes in the *Gata4* mutant mice (data not shown).

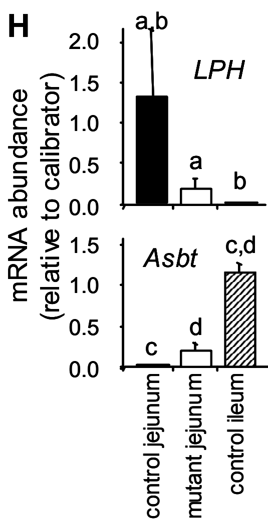
Perhaps the most distinguishable change in the secretory cells is the volume density of goblet cells, which normally increases from duodenum to distal ileum, and is reflected by an increase in goblet cell number on villi and an increase in abundance of small intestinal goblet cell markers such as mucins (39). In the jejunum of *Gata4* mutant mice, the number of periodic-acid Schiff (PAS)-positive goblet cells on villi (Fig. 6A-C) was significantly increased ~40% from 11.3±3.5 goblet cells/villus (mean±SD) in control jejunum to 15.8±2.6 goblet cells/villus in *Gata4* mutant jejunum ($P<0.05$, $n=6$), as determined by unbiased counting. Quantification of mucin-2 (*Muc2*) mRNA, the principal small intestinal goblet marker and indicator of goblet cell volume, revealed an increase toward ileal levels (Fig. 6D), but, like the absorptive enterocytes genes, did not demonstrate a complete transformation to ileal levels. These data show that inactivation of *Gata4* in the adult jejunum results in an increase in the goblet cell population, supporting a partial jejunal-to-ileal transformation in cell fate allocation.



G

normal pattern	target gene	fold change
J>I	*Fabp1*	0.2*
	LPH	0.1*
J=I	*SI*	0.9
	Fabp2	0.6
J<I	*Asbt*	20**
	Ilbp	644***
	Gapdh	

jejunum: control, mutant; No RT



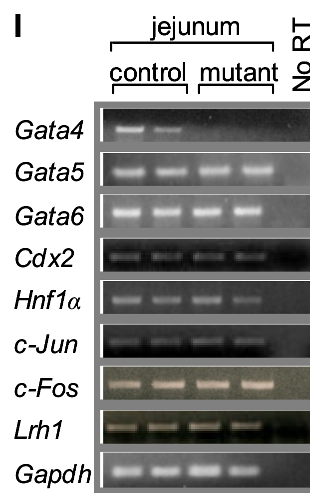


Fig. 5. Absorptive enterocyte gene expression program in jejunum is transformed into an ileal-like pattern in *Gata4* mutant mice. (A-C) Cytoplasmic Fabp1 immunofluorescence (green) and DAPI nuclear staining (blue) of the absorptive enterocytes of control jejunum (A), mutant jejunum (B), and control ileum (C) showing a reduced expression of Fabp1 in mutant jejunum. (D-F) Microvillus membrane Asbt immunofluorescence (green) and DAPI nuclear staining (blue) of control jejunum (D), mutant jejunum (E), and control ileum (F) showing an induction of Asbt in mutant jejunum. (G) Real-time and semi-quantitative RT-PCR conducted on jejunal RNA from control and *Gata4* mutant mice. Real-time RT-PCR (left) is shown as a ratio of mRNA abundance of *Gata4* mutant jejunum as compared to controls (*P<0.05, **P<0.01, ***P<0.001) of genes normally expressed higher in jejunum than ileum (J>I), equally in jejunum and ileum (J=I), and lower in jejunum than ileum (J<I). Semi-quantitative RT-PCR (right) is shown for two representative samples from control and *Gata4* mutant mice. A reaction without reverse transcriptase (No RT) served as a control for DNA contamination. (H) Comparison of LPH and Asbt mRNA in control jejunum, mutant jejunum and control ileum showing that the transformation to an ileal-like phenotype is not complete. (I) Semi-quantitative RT-PCR analysis for *Gata5, Gata6, Cdx2,* hepatocyte nuclear factor-1α *(Hnf1α), c-Jun, c-Fos,* liver receptor homologue-1 *(Lrh1)* and *Gapdh* on RNA from jejunum showing that the mRNA abundance between control and *Gata4* mutant mice are not different. A reaction without reverse transcriptase (No RT) served as a control for DNA contamination.

Enteroendocrine cells are equally distributed throughout the small intestine, but subpopulations are localized to distinct regions based on function. For example, hormones that stimulate pancreatic secretions, such as cholecystokinin (CCK) are synthesized and secreted from enteroendocrine cells in the duodenum and jejunum, whereas hormones that inhibit pancreatic secretions, including peptide YY (PYY) are scarce in the proximal small intestine but more abundant distally. To test the hypothesis that the absence of Gata4 causes a jejunum-to-ileum transformation in enteroendocrine subpopulations, real-time RT-PCR for *CCK* and *PYY* mRNA was undertaken. In the jejunum of *Gata4* mutant mice, *CCK* mRNA was decreased, whereas *PYY* mRNA was increased, both shifting towards ileal levels (Fig. 6E and F). These data suggest that the inactivation of *Gata4* in the adult jejunum results in a partial redistribution of enteroendocrine subpopulations towards an ileal-like composition.

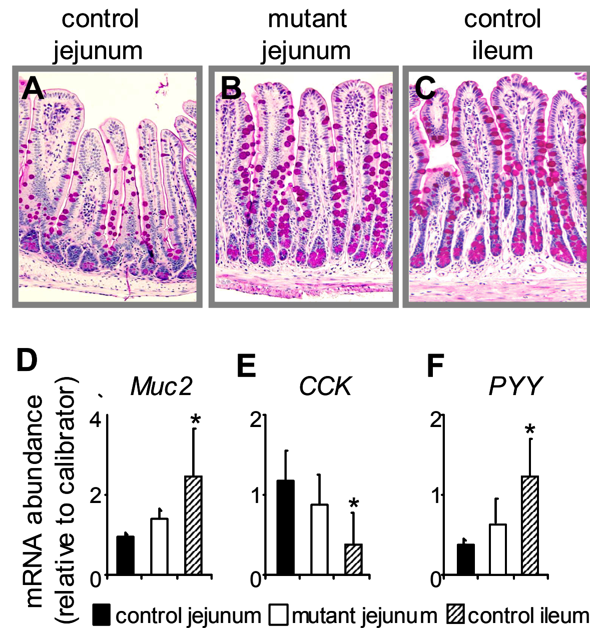


Fig. 6. Secretory lineages are redistributed towards an ileal-like composition. (A-C) Periodic acid Schiff (PAS) staining of control jejunum (A), mutant jejunum (B), and control ileum (C) showing expansion of the goblet cell population in *Gata4* mutant jejunum. (D-F) Real-time RT-PCR of *Muc2* (D), *CCK* (E), and *PYY* (F) mRNA abundance in control jejunum (solid bars), mutant jejunum (open bars), and control ileum (hatched bars) (*P<0.05, as compared to control jejunum, mean±SD).

There was no evidence of abnormal proliferation or apoptosis identified by anti-Ki67 and anti-cleaved caspase3 staining in the jejunum of *Gata4* mutant mice as compared to controls (Fig. 7A-D). This was true not only 2 weeks after the completion of Tamoxifen™ treatment, but also 1, 2, and 3 days after the beginning of Tamoxifen™ treatment (data not shown). We also found no difference in the abundance in the mRNAs for Wnt and Hedgehog signaling proteins, including T-cell factor-4 (*Tcf4*), cyclinD1, bone morphogenetic protein-4, (*Bmp4*), sonic hedgehog (*Shh*), and Indian hedgehog (*Ihh*) between control and *Gata4* mutant mice (Fig. 7E). Together, these data indicate that inactivation of *Gata4* in the small intestine does not appreciably alter proliferation or the normal apoptotic fate of differentiated cells, and likely does not involve Wnt and Hedgehog signaling pathways.

Cell lineage allocation is determined in large part by the Notch signaling pathway, whereby Notch activates *Hes1* in intestinal progenitor cells which, in turn, represses *Math1* resulting in the specification of the absorptive enterocyte cell fate (31). In occasional cells that do not express *Hes1*, *Math1* is expressed resulting in a commitment to the secretory lineages. Inactivation of the *Hes1* gene results in low numbers of absorptive enterocytes and an expansion of the secretory lineages (17) whereas deletion of the *Math1* gene results in an elimination of

secretory cells (46). Expression of neurogenin-3 (*Ngn3*) in *Math1*-positive cells specifies the enteroendocrine lineage. *Notch1*, *Hes1*, and *Ngn3* mRNA abundance was unaffected by the inactivation of *Gata4* as shown by semi-quantitative (Fig. 7F) and real-time RT-PCR (data not shown), but *Math1* mRNA in the jejunum of the *Gata4* mutant mice was significantly increased (P<0.05), and comparable to higher levels of *Math1* mRNA in the control ileum (Fig. 7G), which is consistent with an expansion of the goblet cell lineage.

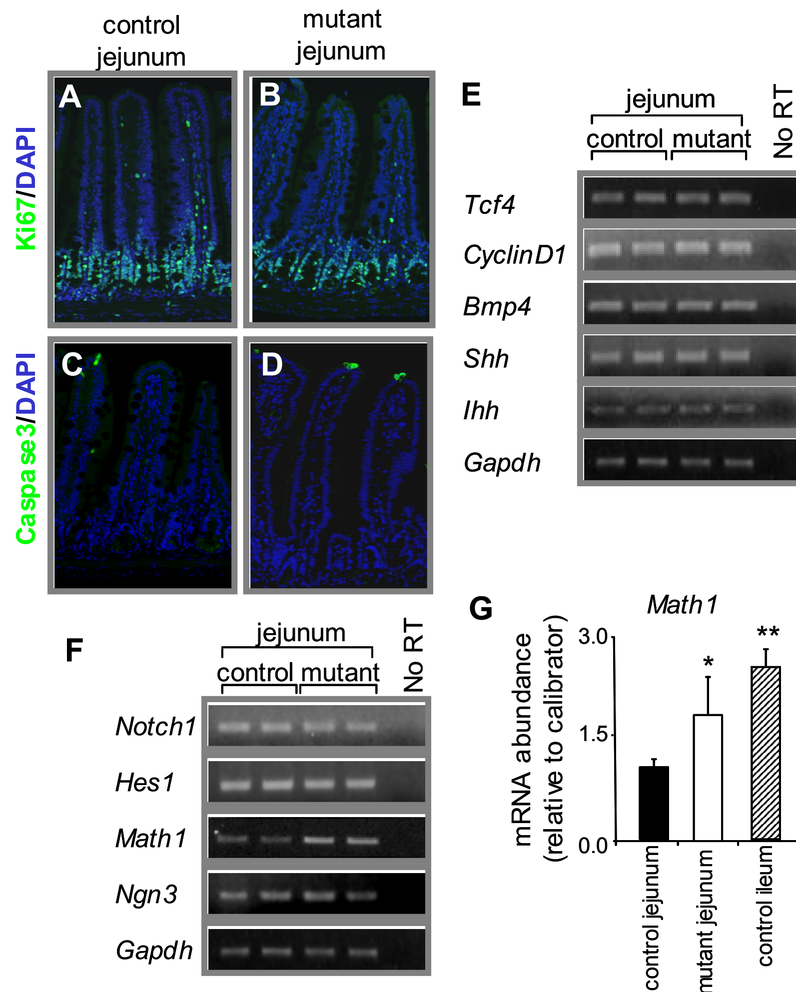


Fig. 7. Proliferation, apoptosis, and signaling pathways in *Gata4* mutant mice. (A,B) Immunofluorescence for the proliferation marker Ki67 (green) and DAPI (blue) showing that the proliferative compartment in the jejunum of control (A) and mutant (B) jejunum were not different. (C,D) Immunofluorescence for cleaved caspase3 (green) and DAPI (blue) showing that cell death in control (C) and mutant (D) jejunum were not different. (E) Semi-quantitative RT-PCR analysis of T-cell factor-4 (*Tcf4*), Cyclin D1, bone morphogenic protein-4 *(Bmp4),* sonic hedgehog *(Shh),* Indian hedgehog *(Ihh),* and *Gapdh* showing that the mRNA abundance between control and *Gata4* mutant mice are not different. (F) Semi-quantitative RT-PCR analysis on *Notch1, Hes1, Math1,* neurogenin3 (*Ngn3*), and *Gapdh* showing that jejunal mRNA abundance between control and *Gata4* mutant mice are not different, with the exception of *Math1*, which is significantly greater by real-time RT-PCR (G) in both mutant jejunum (P<0.05) and control ileum (P<0.01) as compared to control jejunum (mean±SD).

DISCUSSION

Gata4, a member of an evolutionarily conserved transcription factor family, is expressed in multiple tissues throughout development and into adulthood, but due to the embryonic lethality of published *Gata4* knockout models (20, 25, 28, 45, 47), its function in vivo in adults is unknown. In the mature small intestine of mice, Gata4 is expressed in the absorptive enterocytes on villi and in all epithelial cells of the crypt (Fig. 1). In both mice and humans, Gata4 is highly expressed in jejunum, but nearly undetectable in distal ileum (Fig. 2), suggesting a role in the regulation of proximal-distal homeostasis within the intestine. In the present study, we established an inducible, intestine-specific inactivation model in which a truncated, transcriptionally inactive Gata4 mutant is synthesized (Figs 3 and 4). Adult mice expressing mutant Gata4 in place of wild type Gata4 in the jejunum display a partial jejunum-to-ileum transformation manifested by an attenuation of absorptive enterocytes gene normally expressed in jejunum, but not in ileum, an induction of genes not normally expressed in jejunum, but highly expressed in ileum (Fig. 5), and a redistribution of secretory cells toward an ileal phenotype (Fig. 6). Although the jejunum-to-ileum transformation is not complete, indicating that Gata4 is not the only regulatory protein involved in distinguishing jejunum from ileum, these data indicate that Gata4 is necessary, in part, for the maintenance of jejunual-ileal identities in the adult mouse small intestine.

The synthesis of a truncated form of Gata4 in the mutant mice was unexpected. This protein, indicated as Gata4$^{\Delta ex2}$, is transcriptionally inactive, but is able to bind to DNA and act as a dominant-negative Gata factor. Gata4$^{\Delta ex2}$ in mutant mice and Gata4 in control mice are each co-expressed with Gata6 in absorptive enterocytes on villi (Fig 1 and data not shown), and possibly in crypts. Thus, the dominant-negative activity of Gata4$^{\Delta ex2}$ could conceivably mask underlying compensation by Gata6. However, we argue that the same competition for DNA elements that might occur between Gata4$^{\Delta ex2}$ and Gata6 in mutant mice would also occur between Gata4 and Gata6 in control mice, especially since Gata4$^{\Delta ex2}$ and Gata4 are synthesized in similar amounts. We believe that under these conditions, Gata4$^{\Delta ex2}$ would occupy the identical cadre of binding sites in mutant mice as Gata4 would in control mice, and thus would not interfere with normal Gata6 regulation. We therefore conclude that although we are unable to assess the compensatory function of Gata6, the phenotype observed in the present study in the Gata4 mutant mice is highly likely to be reflective of Gata4 function.

Our data indicate that Gata4 is involved in multiple pathways (Fig. 8) including (a) activation of a jejunal gene expression program in absorptive enterocytes, (b) repression of an ileal gene expression program in absorptive enterocytes, and (c) alteration in cell fate specification. Gata4 could act as a global regulator at an early step in cellular differentiation whereby cells of all lineages are fated toward a jejunal phenotype in the presence of Gata4, but toward an ileal phenotype in the absence of Gata4. This hypothesis is supported by the expression of Gata4 throughout the crypt, but does not take into account a function for Gata4 in

absorptive enterocytes on villi. Gata4 could also act via direct effects on promoters in mature absorptive enterocytes to activate or repress specific target genes, and in crypts to regulate cell fate allocation. This hypothesis accounts for a Gata4 function in both absorptive enterocytes on villi and progenitor cells in crypts, and is thus the favored hypothesis at this time.

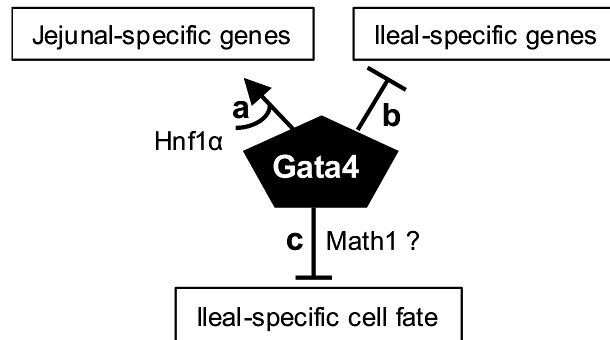


Fig. 8. Model showing that Gata4 maintains a jejunal phenotype through (a) activation of a jejunal gene expression program in absorptive enterocyte, (b) repression of an ileal gene expression program in absorptive enterocytes, and (c) alteration in cell fate specification.

Activation of absorptive enterocyte genes in the jejunum by Gata4 has been hypothesized in our previous work to occur in cooperation with Hnf1α through an evolutionarily conserved mechanism requiring physical interactions between these two transcription factors (43, 44). We have further hypothesized that overlapping expression and subsequent co-regulation by these two transcription factor families is a means to achieve high levels of absorptive enterocyte-specific gene expression (19). Cooperative activation by Gata4 and Hnf1α has been demonstrated in cell culture over-expression experiments not only for *LPH* (19, 43, 44), but also for *Fabp1* (7) and *SI* (3, 19, 43, 44)We found that in the jejunum of adult *Hnf1α*-/- mice, gene expression of both *Fabp1* and *LPH* was attenuated, whereas that of *SI* and *Fabp2* was similar to that in wild-type mice (2). The identical effects in both the adult *Gata4* mutant and *Hnf1α* null mice is supportive of co-regulation by Gata4 and Hnf1α, and is further consistent with our hypothesis that these two transcription factors act in concert to activate a subset of absorptive enterocyte target genes.

Although the induction of ileal genes such as *Asbt* and *Ilbp* in the jejunum of *Gata4* mutant mice is consistent with our hypothesis that Gata4 maintains a jejunal identity, this finding was nevertheless surprising because it indicates that Gata4 is responsible for the repression in the jejunum of ileal-specific genes. Gata4 is generally thought of as an activator of gene transcription, although it may recruit friend of GATA (FOG) cofactors and repress the expression of certain Gata4 target genes(40). Gata1 demonstrates multiple functions in hematopoeisis where it is required for the activation of erythroid genes as well as repression of early hematopoietic genes (30). Using a biotinylated tagging/proteomics approach in mouse

erythroleukemic (MEL) cells, Gata1 was found to co-exist in multiple complexes, and divergent complexes were found to associate in vivo with discrete Gata1 target genes, thereby linking specific Gata1 partners to distinct aspects of its functions. We propose an analogy with Gata1 in that Gata4 can function as both an activator and repressor depending on the context of the promoter and the transcription factors and cofactors with which Gata4 interacts within the cell.

To our knowledge, this is the first report showing an induction in *Asbt* and *Ilbp* gene expression in the adult jejunum. This finding may be clinically relevant for patients who have undergone resection of the terminal ileum or who have suffered disease-related changes in the ileal mucosa, as in cystic fibrosis and Crohn's disease. These patients develop severe bile acid malabsorption characterized by diarrhea and eventually gall stone and kidney stone formation (23). Further characterization of the Gata4-dependent inhibitory pathway that blocks ileal gene expression in the jejunum may reveal specific targets that can be used for therapeutic intervention in order to induce bile acid absorption in the jejunum of patients with impaired ileal function.

The redistribution in the number of goblet cells and type of enteroendocrine cells in jejunum to an ileal-like pattern in the absence of Gata4 indicates that Gata4 plays a role in defining cell fate, and likely explains the function of Gata4 in crypt progenitor cells where this process occurs. *Math1* is expressed in secretory cells on villi as well as isolated progenitor cells in crypts, and is responsible for commitment of the secretory lineages (27, 46). Thus, the increase in *Math1* mRNA in the jejunum of *Gata4* mutant mice is consistent with an expansion of the goblet cell composition on jejunal villi. However, the higher levels of *Math1* mRNA in the jejunum of the *Gata4* mutant mice as well as in control ileum could be a reflection of the increase in goblet cell number, or could represent a novel regulatory pathway whereby Gata4 controls the composition of the secretory cell lineages in jejunum vs. ileum through a Math1-dependent process.

Inactivation of jejunal Gata4 resulted in a ~40% decrease in CCK mRNA and a ~40% increase in PYY mRNA, all within the same set of RNA samples. It is well known that in addition to the specification of enteroendocrine cells by Math1/Ngn3, other factors, including Pax4, Pax6, BETA2/NeuroD, and pancreatic-duodenal homeobox-1 (Pdx1), regulate the specific subsets of enteroendocrine genes along the cephalo-caudal axis (32). Our data suggest that Gata4 is yet another factor involved in distinguishing the regional fate of enteroendocrine subpopulations, specifically between jejunum and ileum. Since Gata4 is not expressed in mature enteroendocrine cells (Fig. 1)(9), it is likely that this process occurs within the progenitor crypt compartment.

Although there are no other known in vivo examples of a regional shift in phenotype within the small intestinal system, there are examples of homeotic shifts in other organs of the gastrointestinal tract. These include Cdx2, which is normally expressed in the small and large intestine, and Pdx1, which is normally expressed in pancreas and duodenum. Ectopic expression of *Cdx2* in the stomach of transgenic mice gives rise to patches of intestinal-like tissue (35),

whereas ectopic expression *Pdx1* in Xenopus transforms part of the liver into pancreas (16). These data demonstrate that specific transcription factors expressed in restricted patterns along the anterior-posterior axis are responsible for the maintenance of regional identities along the gastrointestinal tract, and that Gata4 is one of these factors.

The adult intestinal epithelium is a rapidly renewing tissue with differential functions along the cephalo-caudal axis and therefore constant positional information to newly emerging cells is essential for the maintenance of local functions. Despite significant advances in our understanding of the molecular control governing intestinal differentiation and development, little is known about the molecular signals required for maintenance of proximal-distal identities in adult intestinal homeostasis. Insight into these molecular signals may be of clinical value in situations where local functions are lost due to disease or resection. In the present study, we have now uncovered a member of the well studied GATA family, Gata4, as a major and essential positional signal that is required for the maintenance of differential functions between jejunum and ileum in vivo.

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CHAPTER 5

Gata4 and Hnf1α are partially required for the expression of specific intestinal genes during development

Tjalling Bosse[1], John J. Fialkovich[2], Christina M. Piaseckyj[2], Eva Beuling[3], Henrike Broekman[4], Richard J. Grand[2,5], Robert K. Montgomery[2,5], Stephen D. Krasinski[2,5,6]

[1]School of Medicine, University of Amsterdam, Amsterdam, The Netherlands 1100DD; and [2]Division of Gastroenterology and Nutrition, Department of Medicine, Children's Hospital Boston, Boston, Massachusetts, 02115; [3]School of Medicine, Erasmus University Rotterdam, Rotterdam, The Netherlands, 3000DR; [4]School of Medicine, University of Utrecht, Utrecht, The Netherlands, 3508TC; [5]Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, 02115; and [6]Dorothy R. Friedman School of Nutrition Science and Policy, Tufts University, Boston, Massachusetts, 02111

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. During a narrow window just prior to weaning, 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 (reviewed in (36)). 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 (reviewed in (18)). 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 (reviewed in (28)). 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 (17, 19, 24, 34, 35, 37, 38, 42, 44). 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 (20, 23, 25, 26, 38, 45). 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 hepatocye nuclear factor-1 (Hnf1) and caudal (Cdx) families of homeodomain proteins (5, 6, 8-10, 12, 14, 15, 21, 27, 40, 43, 50). Gata4 and Hnf1α are the predominant family members in nuclear extracts from mouse intestinal epithelial cells that bind to the *LPH* and *SI* promoters (4, 5, 46), and both Gata4 and Hnf1α activate the *LPH, Fabp1*, and *SI* promoters in cell culture over-expression experiments (5, 6, 8, 9, 12, 21, 27, 40, 46). Gata4 and Hnf1α physically associate and synergistically activate the *LPH*, *Fabp1*, and *SI* genes (5, 9, 46) through an evolutionarily conserved pathway (46, 47). We have postulated that this interaction is a means to achieve high levels of intestine-specific gene expression in vivo (20). Gata4 and Hnf1α are co-expressed in the nuclei of epithelial cells of the adult jejunum establishing a topographic basis of co-regulation. Recently, we found that 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 (3). Germline *Hnf1α* knockout mice survive into adulthood and demonstrate sterility, diabetes, delayed growth rate, and liver dysfunction (22). 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* (3, 4). 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 cytodifferentation 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 mouse models were used in the present study, including an inducible, intestine-specific *Gata4* inactivation model (3) and a germline *Hnf1*α null mouse model (4, 22), all in a C56BL/6 background.

To inactivate *Gata4* in the small intestinal epithelium, *Gata4*$^{flox/flox}$ mice (32) were crossed with transgenic Villin-*Cre*ER^{T2} mice (11) 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. Mice were screened for specific genotypes using PCR on tail DNA as previously described (3). 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 (2). Control and *Gata4* mutant animals were sacrificed for study at E17.5, P10, P20, P30 or 6-10 wk (adult). *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 (3).

Hnf1α-/- mice survive into adulthood, but are sterile, requiring mating of *Hnf1α*+/- parents to generate both null and wild-type study animals (22). All mice were genotyped using PCR on tail DNA as previously described (4). *Hnf1α*+/+ control and *Hnf1α*-/- littermates were sacrificed for study at E17.5, P1, P4, P7, P14, P21 or P28.

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 (33).

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 (4, 46). 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 (3, 4). 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 (3). 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.

The primary antibodies used were goat anti-Hnf1α (1:250; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-Gata4 (1:500; 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). 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 (46) from pooled mucosal scrapings of 4 cm segments at the geometric center of the small intestine (mid-jejunum) from P7, P14, P21, P28, and adult (6-12 wk) mice. In selected experiments, nuclear and non-nuclear fractions were isolated from pooled mucosal scrapings of 4 cm segments at the geometric center of the small intestine (mid-jejunum) 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 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 (4) using 20-25 µg of nuclear or non-nuclear extracts. The primary antibodies included affinity-purified goat polyclonal antibodies for Gata4 or Hnf1α (Santa Cruz). 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 (21). These included the Gata binding site present in the Xenopus *Fabp1* promoter (X-GATA, 5'-GGAGATCCCTGTACAGATATGGGGAGAC-3') (16), and the Hnf1 binding site present in the rat β-fibinogen promoter (β-Fib, 5'-CAAACTGTCAAATATTAACTAAAGGGAG-3') (7). 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 (24). 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 *LPH* and *Fabp1*, markers of cytodifferentiation (17, 34), 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 (Fig. 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) (Fig. 1B). These data confirm that *LPH* and *Fabp1* are markers for cytodifferentiation of the mouse midgut.

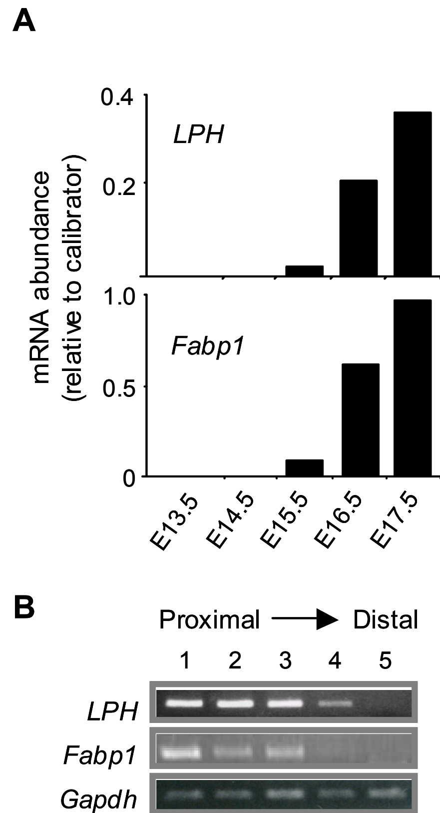

Fig. 1. *LPH* and *Fabp1* mRNAs are induced in the mouse small intestine during cytodifferenation. A. *LPH* and *Fabp1* mRNAs are first detected in mouse small intestine at E15.5. *LPH* and *Fabp1* mRNAs were quantified by real-time RT-PCR in whole intestine of mouse embryos obtained from timed-pregnant mothers. Data were obtained from a single mouse at each age group. 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.

Inactivation of *Gata4* or *Hnf1α* in the jejunum of adult mice results in an attenuation (~90% reduction) of both *LPH* and *Fabp1* gene expression (3, 4). 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 (Fig. 2A). At E17.5, *Gata4* was specifically inactivated in the midgut of *Gata4* mutant mice (Fig. 2B). Body weight, gross intestinal structure, and overall intestinal histology, as indicated by H&E staining, of Gata4 mutant mice was 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% (Fig. 2C, *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 (Fig. 2C, *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.

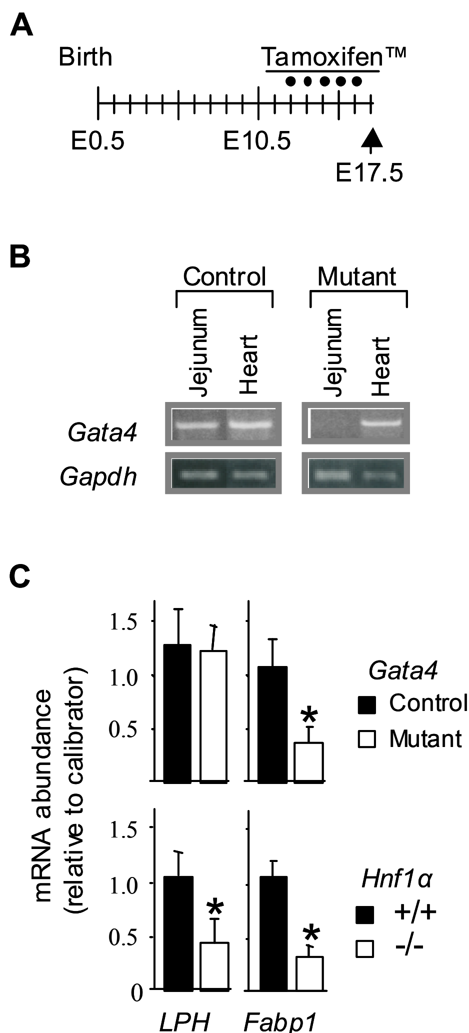

Fig. 2. Gata4 and Hnf1α are differentially required for *LPH* and *Fabp1* gene expression during cytodifferentiation. A. Schematic representation showing that timed-pregnant mothers with litters containing both control (*Gata4*$^{flox/flox}$, Cre negative) and *Gata4* mutant (*Gata4*$^{flox/flox}$, Cre positive) mice 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 obtained from the midgut 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 jejunum (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.

To further understand the regulation of target genes by Gata4 and Hnf1α during cytodifferentiation, real-time RT-PCR was used to quantify the expression patterns of these transcription factors during this time-frame more completely. *Gata4* and *Hnf1α* mRNAs were

expressed throughout cytofifferentiation, with the highest levels occurring at E17.5 (Fig. 3A). 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 demonstrated a generally increasing proximal-to-distal gradient, as shown by semi-quantitative RT-PCR (Fig. 3B). Gata4 and Hnf1α protein were co-localized on nascent villi and intervillus regions of the midgut at E16.5 (Fig. 3C-E), and were specifically localized to the nucleus at this age (Fig. 3F-H), as previously shown for Gata4 (30). Together, these data demonstrate a topographic basis for possible co-regulation by Gata4 and Hnf1α during cytodifferentiation.

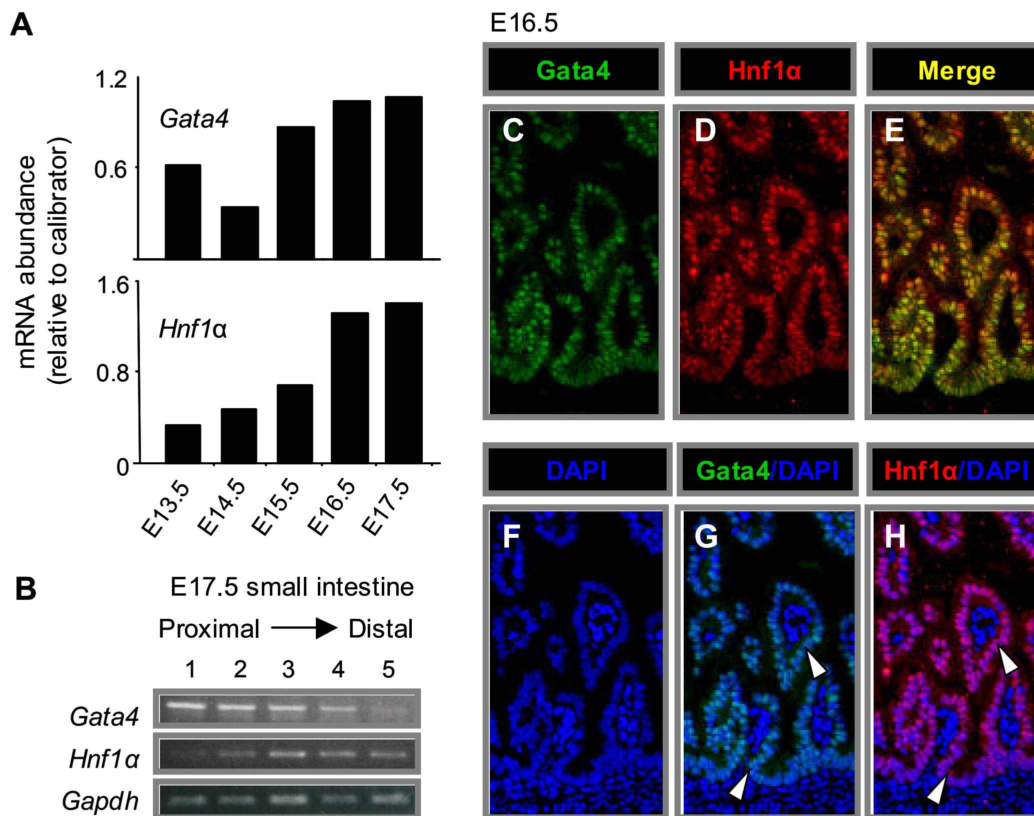

Fig. 3. Gata4 and Hnf1α are co-expressed in the nuclei of small intestinal epithelial cells during cytodifferentiation. A. *Gata4* and *Hnf1α* mRNAs demonstrate an increasing pattern during cytodifferentiation. *Gata4* and *Hnf1α* mRNA was quantified by real-time RT-PCR in whole intestine of mouse embryos 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 the nuclei of epithelial cells of midgut (segment 3) at E16.5. Immunofluorescence was conducted using rabbit anti-Gata4 (C,E,G) and goat anti-Hnf1α (D,E,H), and nuclei were revealed using DAPI (F-H) as indicated. Co-localization of Gata4 and Hnf1α in nuclei are indicated by merged photomicrographs as indicated (E,G,H). *White arrowheads* indicate nuclei.

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 (reviewed in (18)). 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 (2) (Fig. 4A) 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) (Fig. 4A, *arrows*). *Gata4* was expressed normally in heart, liver and stomach (data not shown), but was absent in the jejunum at all ages (Fig. 4B), verifying the Gata4 inactivation model for the study of postnatal development.

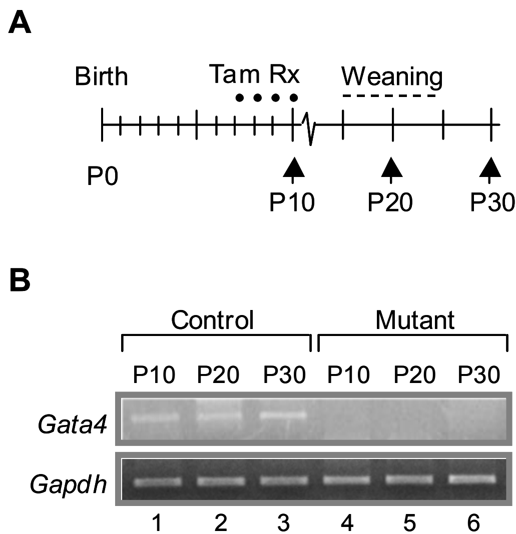

Fig. 4. *Gata4* is inducibly inactivated in the mouse jejunum. A. Schematic representation showing that control (*Gata4*$^{flox/flox}$, Cre negative) and *Gata4* mutant (*Gata4*$^{flox/flox}$, Cre positive) postnatal mice were treated with 4 daily injections of Tamoxifen™ (*filled circles*) beginning at P7. Mice were sacrificed for analysis at P10, P20, and P30 (*arrows*). The weaning transition occurs from P14 – P21, indicated by the *dotted line*. B. *Gata4* is inactivated in the jejunum of *Gata4* mutant mice. 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.

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, Fig. 5A, *top*), a difference that is similar to that in adult mice (3). *Fabp1* mRNA levels were reduced by the inactivation of Gata4 ~50% at P10 (P<0.05, Fig. 5A, *middle*), similar to that observed at E17.5 (see Fig. 2C). *Fabp1* mRNA abundance in Gata4 mutant mice at P20 and P30 was <10% of that in control mice (P<0.05, Fig. 5A, *middle*), similar to that in adult mice (3). *SI* mRNA levels revealed an expected increase during weaning, but no difference between control and *Gata4* mutant mice at any postnatal time-point (Fig. 5A, *bottom*).

Immunofluorescence for LPH, Fabp1, and SI in control and Gata4 mutant mice during weaning followed the results of their mRNA abundances. LPH immunoflouresence was specific to the microvillus membrane in the P10 jejunum (Fig. 5B), and was not affected by the inactivation of *Gata4* (Fig. 5C), consistent with mRNA levels. LPH immunofluorescence was also present in the jejunum of P30 control mice (Fig. 5D), but absent in the *Gata4* mutant mice (Fig. 5E), 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 (Fig. 5F-I), consistent with its mRNA levels. SI was not detected at P10 in either control or *Gata4* mutant mice (Fig. 5J and K), and was localized to the microvillus membrane at P30 with no apparent difference in intensity between control and *Gata4* mutant mice (Fig. 5L and 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.

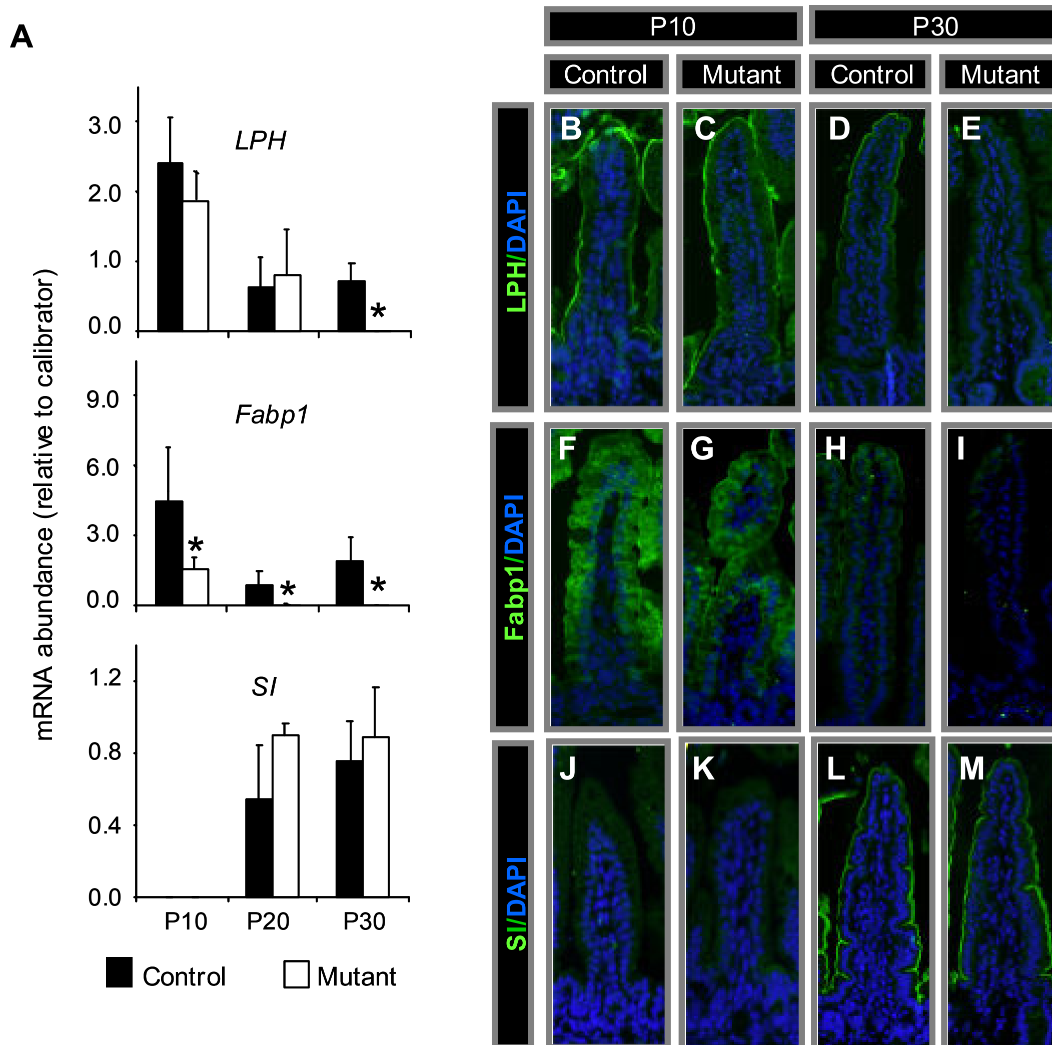

Fig. 5. **Inactivation of *Gata4* in jejunum reveals gene-specific, developmental regulation.** A. *LPH*, *Fabp1*, and *SI* mRNA abundances are differentially affected by the inactivation of *Gata4* during development. Real-time RT-PCR for *LPH* (*top*), *Fabp1* (*middle*) and *SI* (*bottom*) mRNAs were 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 is consistent with mRNA levels.

In *Hnf1α* -/- mice, *LPH* 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 (Fig. 6A, *top*), similar to that in adult *Hnf1α* null mice (4). *Fabp1* mRNA was reduced ~50% in the *Hnf1α* null mice at P7 and P14, similar to that at E17.5 (see Fig. 2C), and was barely detectable at P21 and P28 (Fig. 6A, *middle*), similar to that in adults (4). *SI* mRNA increased during weaning in *Hnf1α*+/+ and *Hnf1α*-/- mice with no significant difference between the two groups (Fig. 6A, *bottom*).

LPH immunofluorescence was most intense in the P7 jejunum of wild-type mice (Fig. 6B), and was consistently less intense in the P7 jejunum of *Hnf1α* null mice (n=3, Fig. 6C), in agreement with the decrease in *LPH* mRNA levels at this age. LPH immunofluorescence was present in the P28 jejunum of wild-type mice (Fig. 6D), but was not detectable in the jejunum of P28 *Hnf1α* null mice (Fig. 6E), again consistent with mRNA levels. Fabp1 immunofluorescence was reduced in *Hnf1*α null mice at both P7 and P28, consistent with its mRNA levels (Fig. 6F-I). SI immunofluorescence was not detected in either control or *Hnf1α* null mice at P7 (Fig. 6J and K), and was similarly intense in the jejunum of both mice at P28 (Fig. 6L and M), correlating with its mRNA levels. These data indicate that Hnf1α, although indispensable for *LPH* and *Fabp1* gene expression after weaning (4), 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.

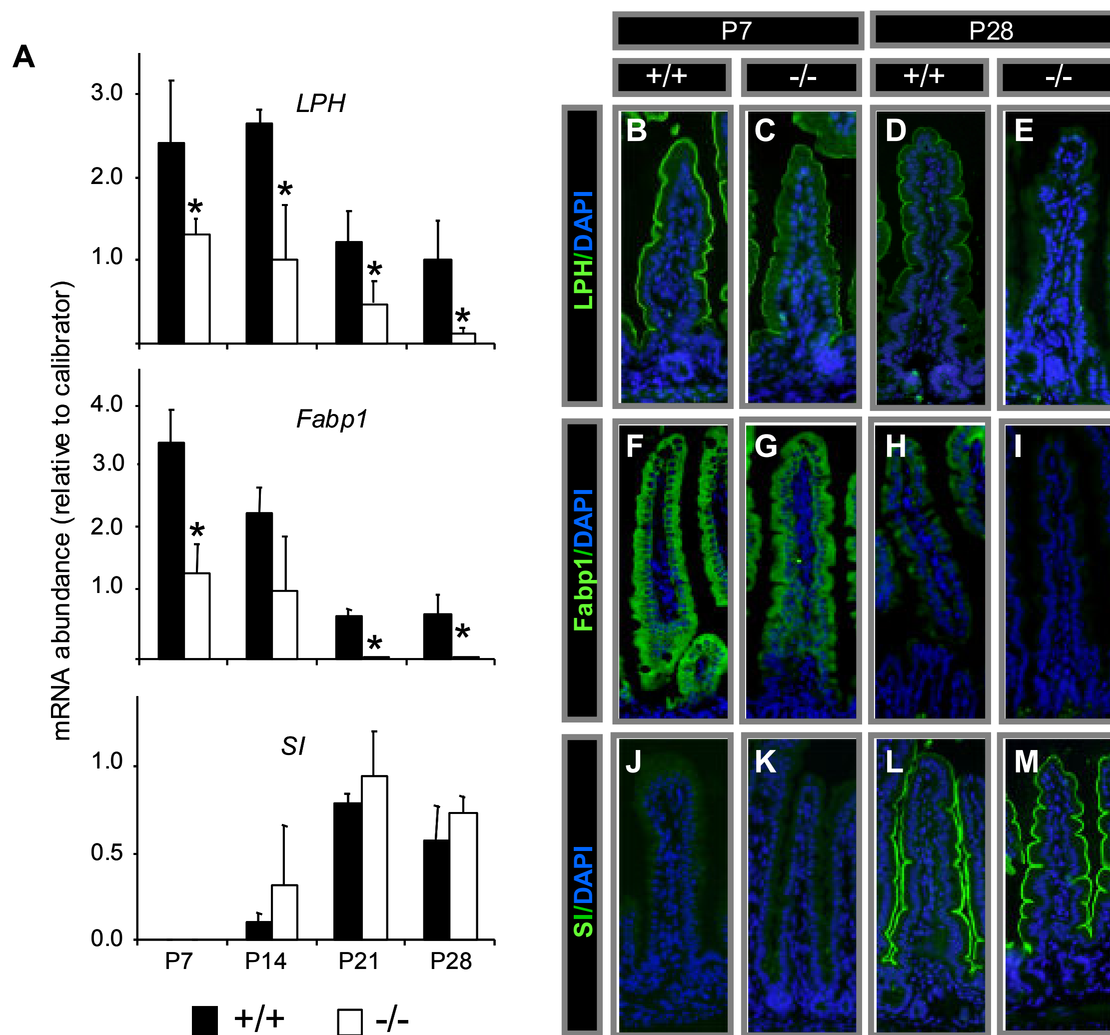

Fig. 6. Null expression of *Hnf1α* demonstrates gene-specific, developmental regulation. A. *LPH*, *Fabp1*, and *SI* mRNA abundances are differentially affected by null expression of *Hnf1α* during development. Real-time RT-PCR for *LPH* (*top*), *Fabp1* (*middle*) and *SI* (*bottom*) mRNAs were conducted 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) mice is consistent with mRNA levels.

Nuclear Gata4 and Hnf1α are paradoxically lost prior to weaning - 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 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) (Fig. 7A, *upper*). *Hnf1α* mRNA was highest at P7, and was significantly lower at all time-points thereafter (Fig. 7A, *bottom*). These patterns are generally consistent with recently published data (13).

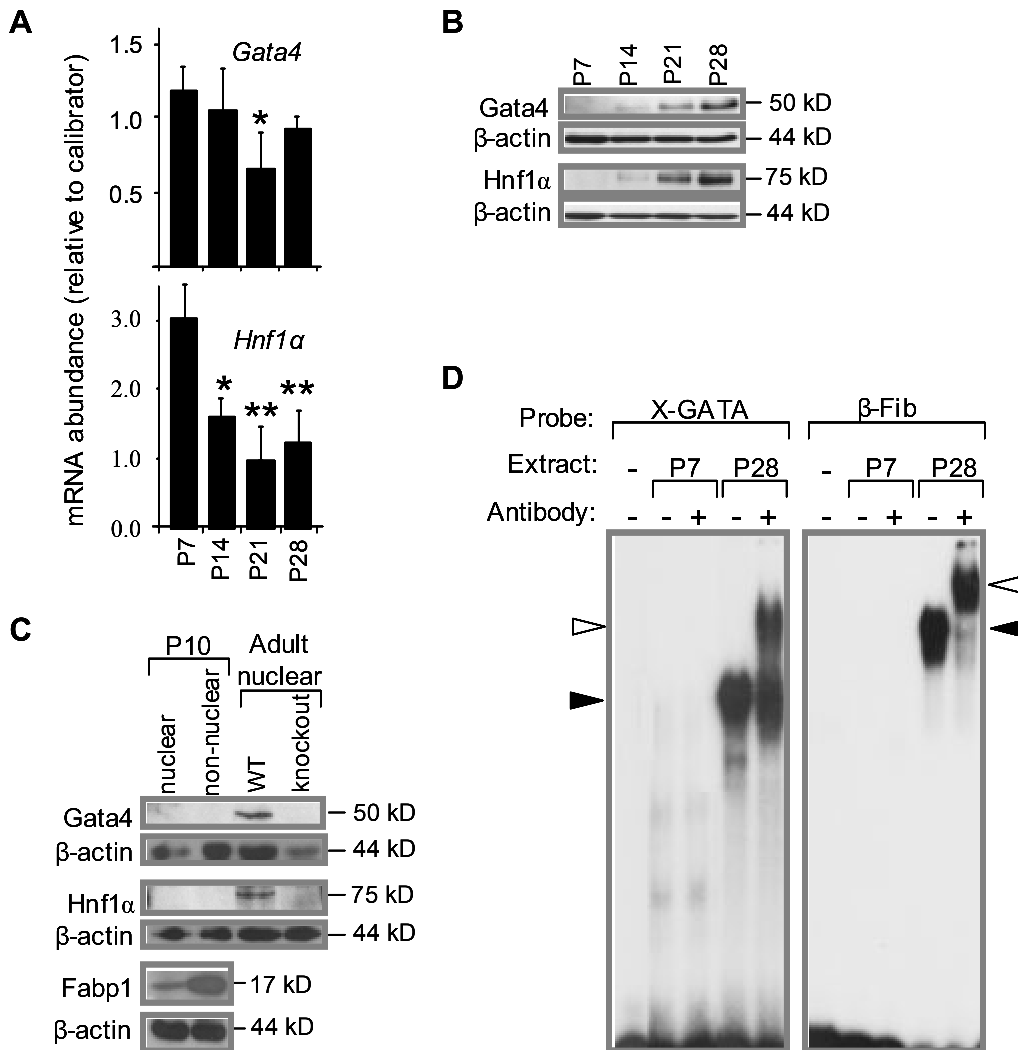

Fig. 7. *Gata4* and *Hnf1α* mRNA abundance do not correlate with their nuclear protein levels during postnatal development. A. *Gata4* and *Hnf1α* mRNA abundance decreases during the weaning transition. *Gata4* and *Hnf1α* mRNA was quantified in wild-type jejunum (segment 3) at P7, P14, P21, and P28 by real-time RT-PCR. Data are mean ± SD of n = 3-5 mice. *P<0.05, as compared to P7 as determined by the Tukey-Kramer multiple comparison test. 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. Blots were re-probed with a β-actin antibody to demonstrate similar protein loading in each lane. C. Gata4 and Hnf1α were not detected in nuclear or 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. Fabp1 immunoblot was conducted to verify the enrichment of cytoplasmic proteins in the non-nuclear fractions. D. 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.

To determine if the mRNA abundance correlates with nuclear protein levels, Western blot analyses using nuclear extracts from mid-jejunum of wild-type mice were performed. Gata4 and Hnf1α were surprisingly undetectable at P7 and very low at P14, but increased markedly at P21 and P28 (Fig. 7B), similar to that previously shown (5), and sharply contrasting with their decreasing mRNA levels during this time interval (see Fig. 7A). The differential abundance of nuclear Gata4 and Hnf1α between preweaning and postweaning jejunum was replicated in at least 2 additional sets of mice. To determine if Gata4 and Hnf1α are localized outside of the nucleus before weaning, Western analyses were performed on nuclear and non-nuclear fractions isolated from jejunal enterocytes at P10. As shown in Fig. 7C, neither Gata4 nor Hnf1α was detected in either the nuclear or non-nuclear fractions at this age. As controls, both Gata4 and Hnf1α were readily detected in nuclear extracts of wild-type jejunum from adult mice, but not in the mature jejunum of the respective knockout models. The relative abundance of Fabp1 in the non-nuclear fraction verifies the enrichment of cytoplasmic protein in this fraction. The inability to detect Gata4 or Hnf1α in nuclear and non-nuclear fractions before weaning was replicated on pooled samples of P7 mouse jejunum. Nuclear extracts from both P7 (Fig. 7D) and P10 (data not shown) were further tested by EMSAs using well characterized DNA binding sites for Gata (16) and Hnf1 (7) transcription factors. Although nuclear extracts from P28 mouse jejunum isolated in parallel showed specific complexes (*filled arrowheads*) that supershifted (*open arrowheads*) with specific antibodies, neither complex could be detected in nuclear extracts from P7 jejunum. Together, these data indicate that Gata4 and Hnf1α are absent, or present at very low levels, in the epithelial nuclei before weaning.

To define the expression patterns of Gata4 and Hnf1α at the cellular level during weaning, and to verify the attenuated levels of Gata4 and Hnf1α before weaning, immunofluorescence experiments were conducted throughout this time interval. Gata4 and Hnf1α were localized to the nucleus of crypt and villus epithelium at P1 (data not shown) and P4 (Fig. 8A-C), revealing a similar pattern to that at E16.5 (see Fig. 3C-H). Neither Gata4 (Fig. 8D and E) nor Hnf1α (Fig. 8F and G) was detected in the nuclei of P7 jejunum. Positive nuclear immunofluorescence for Cdx2 (Fig. 8H and I) in serial sections served as a positive control for nuclear immunostaining (*white arrowheads*) at this age. At P14, Hnf1α was not detected (data not shown), whereas Gata4 was detected only in the nuclei of cells in the crypts and lower villi (Fig. 8J and K). At P21, Gata4 was expressed throughout the nuclei of the crypt and villus epithelium (data not shown), whereas Hnf1α was expressed in the nuclei of cells in the crypt and lower half of the villi, but was absent in the cells at the villus tip (Fig. 8L and M). At P28, Gata4 and Hnf1α were expressed throughout the nuclei of the crypt and villus epithelium (data not shown), similar to that 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 Fig. 7A), but their protein products are expressed at low levels during suckling, specifically beginning the second week of life, just prior to the weaning transition.

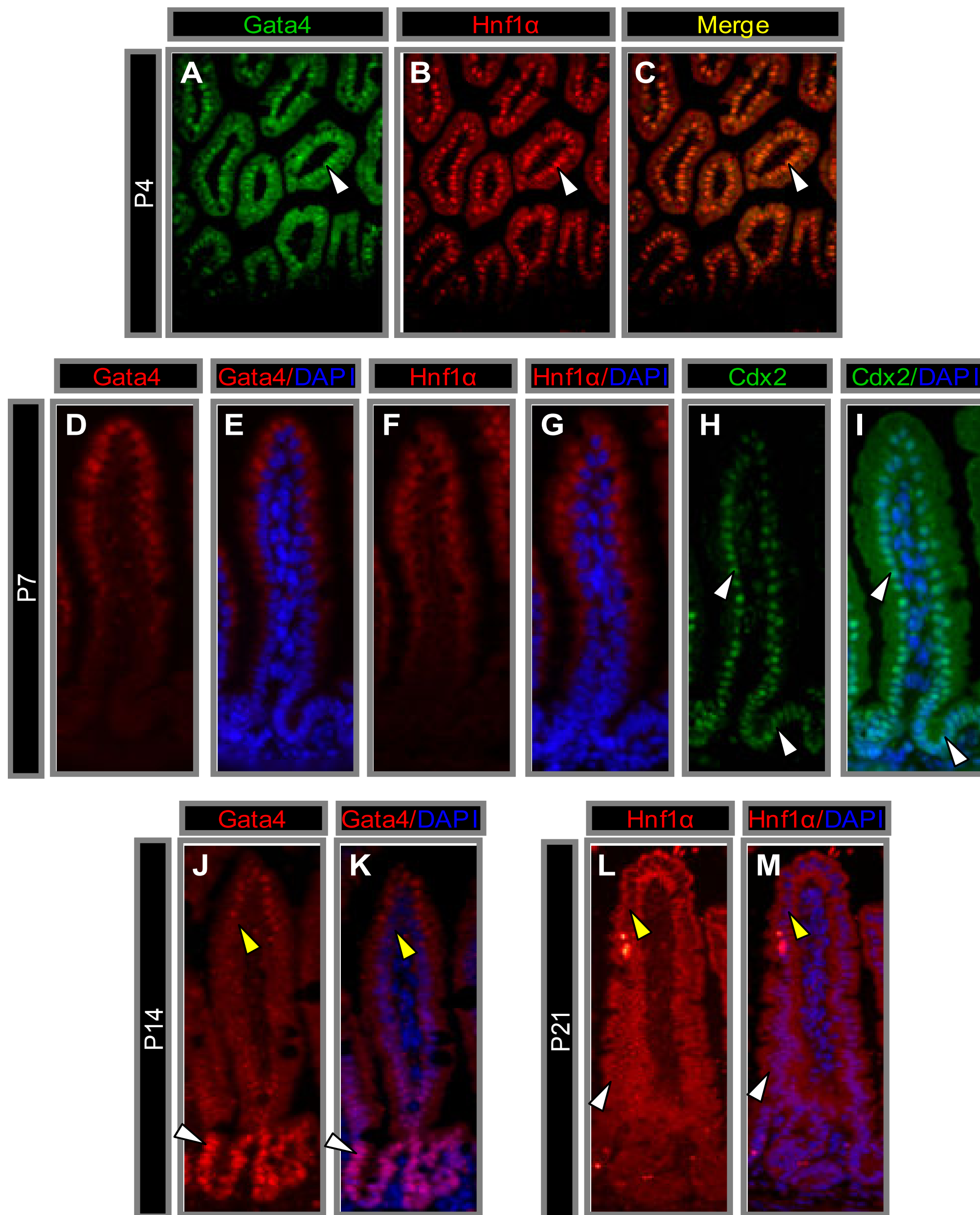

Fig. 8. Gata4 and Hnf1α expression in jejunum is attenuated before weaning. A-C. Immunofluorescence in P4 jejunum using rabbit anti-Gata4 (A), goat anti-Hnf1α (B) and a merged photomicrograph (C). D-I. Immunofluorescence in P7 jejunum using goat anti-Gata4 (D,E), goat anti-Hnf1α (F,G) and rabbit anti-Cdx2 (H,I) along with DAPI nuclear stain (E,G,I). J-M. Immunofluorescence in P14 jejunum using goat anti-Gata4 (J,K), and in P21 jejunum using goat anti-Hnf1α (L,M) without (J,L) and with (K,M) DAPI nuclear stain. *White arrowheads* show specific nuclear immunofluorescence and *yellow arrowheads* indicate the absence of specific nuclear immunofluorescence.

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 (24). However, because this induction is characterized by a 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 (31). To define the possible role of Gata4 as well as Hnf1α in mediating the glucocorticoid response in preweaning mice, we characterized the dexamethasone-mediated response in the context of null intestinal expression of *Gata4* or *Hnf1α*. As shown in Fig 9A (*top*), *SI* mRNA abundance was similarly induced ~80-fold 24 h after dexamethasone administration in wild-type, *Gata4* mutant, and *Hnf1α*-/- mice suggesting that neither Gata4 nor Hnf1α mediates the dexamethasone effect 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 (Fig. 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 (Fig. 9B) after dexamethasone treatment, similar to that which occurs after weaning. Taken together, these data indicate that Gata4 and Hnf1α expression profiles change according to a precocious maturation process induced by glucocorticoids.

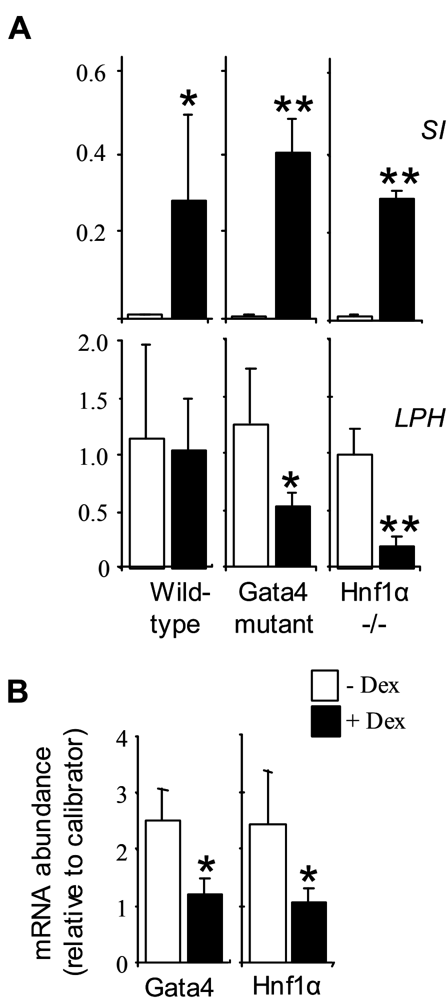

Fig. 9. Dexamethasone induces a precocious weaning response in jejunum in the presence or absence of Gata4 or Hnf1α. A. Dexamethasone induces SI mRNA levels in preweaning mice independently of Gata4 or Hnf1α, but attenuates LPH mRNA levels specifically in the absence of Gata4 or Hnf1α. Mice were treated 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.

DISCUSSION

The establishment of a fully functioning mature mammalian gut is the result of a series of ordered developmental transitions (reviewed in (36)). 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 (reviewed in (18)). LPH, Fabp1 and SI are well characterized markers of these transitions (17, 19, 24, 34, 35, 37, 38, 42), and the Gata and Hnf1 families of transcription factors have been implicated in their regulation (5, 6, 8-10, 12, 15, 21, 27, 40, 50). We have previously shown in mature jejunum that inactivation of either *Gata4* or *Hnf1*α results in a nearly complete attenuation of *LPH* and *Fabp1* gene expression, but no effect on *SI* gene expression (3, 4).

In the present study, we found that the regulation of target genes by Gata4 and Hnf1α during development differs from that in adults (Fig. 10). Before weaning, Gata4 and Hnf1α are only partially required for *LPH* and *Fabp1* gene expression, and their patterns of regulation differ for each of these genes during this time-frame. 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 (9). We further found that Gata4 and Hnf1α are dispensable for *SI* gene expression throughout development. During the suckling period, there is 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 Gata4 or Hnf1α do not 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. These findings suggest that redundant or overlapping mechanisms maintain the expression of *LPH* and *Fabp1* during this time interval. 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 redundant mechanism for the maintenance of gene expression during this critical developmental time interval is a plausible hypothesis. These data also suggest 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 (5, 9, 10, 12, 14, 15, 21, 29, 41, 43, 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.

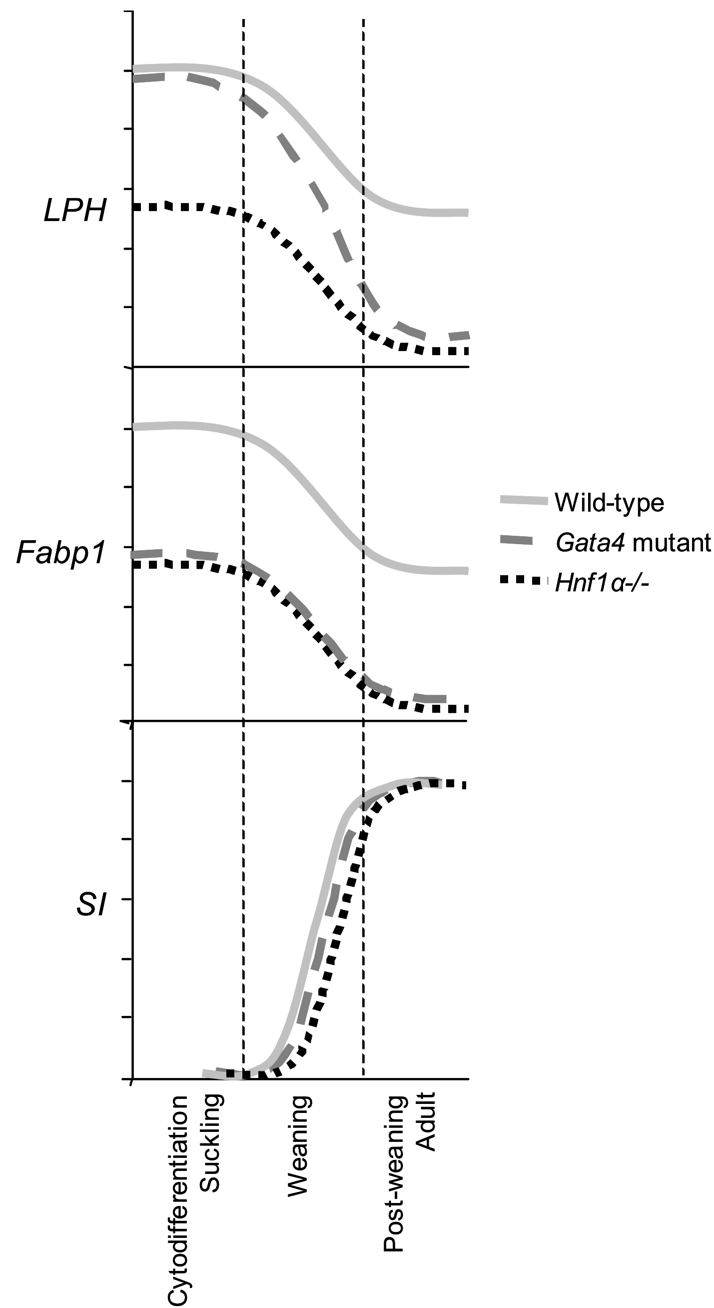

Fig. 10. Summary of Gata4 and Hnf1α regulation of LPH, Fabp1 and SI gene expression during intestinal development. Schematic representation of *LPH* (*top*), *Fabp1* (*middle*), and *SI* (*bottom*) gene expression in wild-type, Gata4 mutant, and Hnf1α-/- during development.

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 (Fig. 1,5 and 6, and (17, 19, 34, 35, 38)), and their promoters contain binding sites for similar sets of transcription factors (8, 21). However, although both are similarly regulated by Gata4 and Hnf1α in mature intestine (3, 4), 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 *Hnf1*α 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 (5, 21). In addition, the abundance of nuclear Gata4 and Hnf1α proteins in the jejunum increases during the weaning transition, paralleling SI gene expression (Fig. 8 and (5, 21)). Despite these compelling data for a combinatory role for Gata4 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 (3, 4). 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.

In our studies, we identified a paradoxical loss of Gata4 and Hnf1α protein in the nuclei of absorptive enterocytes during a narrow window just prior to the onset of weaning. At P7-P14, Gata4 and Hnf1α were greatly reduced in the nuclear fraction of jejunal extracts as determined by Western analysis (Fig. 7B and C) and EMSA (Fig. 7D), and neither could be detected in the nuclei of villus enterocytes in the jejunum by immunofluorescence (Fig. 8). This reduction in Gata4 and Hnf1α nuclear protein occurs over a narrow time-frame at the end of the first week (P4-P7), and regenerates from crypts during the third week of life (Fig. 8). Interestingly, *Gata4* and *Hnf1*α mRNA abundance remains high during this time interval (Fig. 7A) 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 being transcribed, and/or that their translation products are being actively catabolized. However, because Gata4 and Hnf1α are partially regulatory during this time interval, we hypothesize that at least some Gata4 and Hnf1α is normally present in the nucleus, but below the detection limits of our assays. Further, regulation in the knockout models at P7-P14 (Fig. 5 and 6) is virtually identical to that which occurs at E17.5 (Fig. 2), when Gata4 and Hnf1α are normally present in epithelial nuclei, suggesting that the loss of nuclear Gata4 and Hnf1α before weaning is not a critical regulatory

mechanism for *LPH* and *Fabp1* gene expression. We believe that the process of nuclear Gata4 and Hnf1α loss in the second week of life 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 is not understood (1, 51). Characteristic of this process is a dramatic increase in *SI* mRNA abundance 24 h after the administration of glucocorticoids (24). Since the induction in *SI* mRNA is not apparent within the first 8 h (24), 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 (1). Recently, Oesterreicher and Henning (31) 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 (Fig. 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 (Fig. 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 (Fig. 9B), which is also consistent with a maturation of the intestine as *Gata4* and *Hnf1α* mRNAs decline normally during weaning (Fig. 7A). 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 trancriptionally inactive, truncated form of Gata4 (mutant Gata4) that is missing its N-terminal activation domains, but contains its functional zinc finger region (3). Since mutant Gata4 continues to bind DNA, it has the potential to act as a dominant-negative Gata factor (3) masking the activity of other enterocyte Gata factors, such as Gata6, which is co-expressed with Gata4 in villus enterocytes (3, 9, 39). 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 (46). 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 (46, 47), 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 specifically Gata4 function, a masked Gata6 function, and/or a function independent of Gata4/Hnf1α interactions. 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. Precedence for such a mechanism is shown in the liver where embryonic, but not postnatal re-expression of Hnf1α is capable of re-activating the silent phenylalanine hydrolxylase gene in Hnf1α-deficient hepatocytes (49). 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 (3, 4), these transcription factors are only partially required for the same genes prior to the final maturation that occurs at weaning, 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 6

Gata4 dependent repression of ileal gene expression is mediated through FOG interactions

(Manuscript in preparation)

Tjalling Bosse[1], Eva Beuling[2], Daan aan de Kerk[1], Christina M. Piaseckyj[3], Y. Fujiwara[3,4], Stephen D. Krasinski[3,5]

From the[1]Department of Pediatrics, University of Amsterdam, Amsterdam, The Netherlands 1100DD; [2]Erasmus University Rotterdam, Department of Pediatrics, Erasmus MC/Sophia children's hospital, Rotterdam, The Netherlands; [3]Division of Gastroenterology and Nutrition, Department of Pediatrics, Children's Hospital, Boston, Massachusetts, 02115; [4]Department of Hematology and Oncology, Children's Hospital Boston, Massachusetts 02115, USA and [5]Dorothy R. Friedman School of Nutrition Science and Policy, Tufts University, Boston, Massachusetts, 02111

ABSTRACT

GATA-family transcription factors are critical for the differentiation and development of diverse tissues. In the small intestine, Gata4 has been demonstrated to be critical in the maintenance of jejunal-ileal identities. Inactivation of Gata4 leads to an increase in goblet cells, attenuation of jejunal genes (such as lactase phlorizin-hydrolase, LPH) and repression of ileal genes (such as apical sodium bile-acid transporter, Asbt) in the adult mouse jejunum. In this report we use *Gata4* mutant mice to show that Gata4 is a critical factor in the establishment of the horizontal distribution of Asbt gene expression in adult mice, by repressing Asbt expression in the proximal and mid-jejunum during weaning. This Gata4 dependent repression acts through the first 1.1kb of the *Asbt* 5'-flanking region as shown by transient transfections in CT-26 cells. In other organ systems GATA factors function has been shown to be modulated by interactions with other regulators, specifically by the interaction with Friend of GATA (FOG) co-factors. We therefore hypothesized that intestinal Gata4 functions may be mediated by intestinal FOG co-factors. We first determined the expression profile of *Fog1* and *Fog2* in the small intestine and found that *Fog1* is the principal FOG co-factor in the adult small intestine. As an experimental model to search for a requirement for FOG co-factors in the intestinal Gata4 function, we established a mouse line that upon tamoxifen™ induction harbors a single amino acid replacement in Gata4 that impairs its physical interaction with FOG co-factors only in the intestine (*Gata4* knock-in). No gross abnormalities could be identified in the overall structure of the epithelium of these mice, in particular we could not detect an increase in goblet cell population. To determine the role of FOG co-factors in the Gata4 dependent gene expression, mRNA levels of *LPH* and *Asbt* were compared between wild-type, mutant and knock-in jejunum and wild-type ileum. This revealed that FOG co-factors facilitate in the Gata4 dependent repression of *Asbt* in the adult jejunum, however do not play a role in the activation of *LPH* by Gata4. Therefore we infer that the interaction of FOG co-factors, presumably Fog1, with Gata4 in the adult jejunum is necessary for the complete Gata4 repression of ileal-specific gene expression.

INTRODUCTION

This manuscript is in preparation, however the experiments presented were carried out during the process of the thesis preparation and demonstrate significant results with respect to the experiments described in this thesis and therefore were appropriate to include.

Gata4 is a member of an evolutionarily conserved zinc finger containing transcription factor family that plays a crucial role in the maintenance of jejunal-ileal identities in the adult mouse small intestine through a variety of molecular pathways (3). In the adult small intestine, Gata4 is normally expressed in jejunum, but is greatly reduced or absent in distal ileum(3, 36). Recently we showed that mice harboring an intestinal inactive Gata4 mutant have a jejunal phenotype with ileal characteristics. The jejunal epithelium of these mice had an increase number of goblet cells and a redistribution of enteroendocrine subtypes towards an ileal composition, implicating a role for Gata4 in cell-lineage allocation along the horizontal axis. The jejunum also displayed an attenuation in the expression of genes normally highly expressed in the jejunum, such as liver fatty-acid binding protein (*Fabp1)* and lactase-phlorizin hydrolase (*LPH)*, indicating a role for Gata4 in the activation of these genes. The jejunum of these *Gata4* mutant mice further showed a significant induction of the ileal specific genes, ileal sodium bile-acid transporter (*Asbt*) and ileal lipid binding protein (*Ilbp*). The mechanism of activating jejunal genes like *LPH* and *Fabp1* has been extensively studied in cell-culture systems and is likely a result of the physical interaction of the C-terminal zinc finger of Gata4 with the homeodomain region of hepatocytes nuclear factor (Hnf1α)(36). The molecular mechanism involved in the Gata4 dependent repression of ileal genes in the adult jejunum and the role of Gata4 in cell-fate determination however remains to be elucidated. Together these data present the paradigm that Gata4 acts as an activator and a repressor at the same time in the absorptive enterocytes of the adult jejunal epithelium. We hypothesized previously that this duality may be coordinated by specific interactions with other semi-restricted transcription factors or co-factors in the context of the specific promoter sequences.

Friend of GATA (FOG) is a recently discovered multi-zinc finger co-factor family that physically associates with the N-terminal zinc finger of GATA factors, and mediates GATA function. Depending on the context, FOG co-factors can act to further activate or repress the GATA function of target genes (17, 24, 34, 35). The GATA:FOG interaction is conserved in *Drosophila* where the FOG homologue, *U-shaped*, physically associates with *pannier*, a GATA homologue indicating that this interaction is evolutionarily conserved and therefore fundamentally important (10, 15). Two members of the FOG family, Fog1 and Fog2, have been described based on protein structure and expression patterns (31, 32). Fog1 is co-expressed with Gata1 in erythroid and megakaryocytic cells, whereas Fog2 is co-expressed with Gata4 in cardiac tissue. Germline knock-out mice of Gata1 or Fog1, as well as a knock-in of a mutation into Gata1 that disrupts interactions with FOG co-factors, all result in embryonic lethal phenotypes characterized by failure during hematopoiesis (8, 13, 34). Likewise, germline knock-

out mice of Gata4 or Fog2, as well as a knock-in mutation in Gata4 that disrupts FOG interactions, all result in embryonic death due to failure during early cardiogenesis (9, 21, 25, 30, 33). Noteworthy in all of these examples, however, is that FOG co-factors mediate some, but not all GATA functions, indicating that GATA factors have some intrinsic (FOG-independent) activity.

Expression of FOG co-factors in the small intestine has not been reported, however in a recent study evidence was provided for a role of FOG co-factors in the gastrointestinal system. In this study the developing stomach epithelium of *Gata4* knock-in (*Gata4$^{ki/ki}$*) embryos (at E11.5) was analyzed (18). This study showed that in wild-type embryos, *Fog1* is specifically co-expressed with *Gata4* in the developing hindstomach and absent in the forestomach. *Fog2* expression was not evident in the gastric epithelium during fetal or postnatal development, implicating Fog1 as the principal FOG co-factor in the stomach. The stomach epithelium of the *Gata4$^{ki/ki}$* embryos did not show any gross abnormalities, however a significant induction of the endoderm derived signaling molecule sonic hedgehog (*Shh*) was noted in the hindstomach (normally expressed in the forestomach). The findings in the stomach of these Gata4$^{ki/ki}$ mice support a role for the Gata4:FOG interaction in specifying the differences between fore- and hindstomach during early embryogenesis. This study was the first to establish a role for a member of the FOG family, presumably Fog1, in the gastrointestinal system.

In the current study we hypothesized that some of Gata4 functions in the adult small intestine may also be mediated by interactions with members of the FOG family. The expression of the GATA and FOG family overlap in a variety of organ systems, however the expression of FOG family members in the adult small intestine has not yet been analyzed. In the differentiation and development of the cardiac, gonadal, stomach and heamatopoetic system it has been well established that some critical GATA functions are mediated through the interaction with FOG co-factors. In this study we show that Gata4 plays a critical role in determining the proximal boundary of Asbt expression during postnatal development of the small intestine. We further show that 1.1kb of the mouse *Asbt* 5'-flanking region contains the necessary information for the Gata4 dependent repression. In addition we show for the first time that the FOG family members, *Fog1* and *Fog2*, are expressed in the adult small intestine, providing a topographical basis for interactions with Gata4. We further define the role of the Gata4:FOG interaction in the adult small intestine, by establishing an experimental mouse model in which a mutated Gata4 that is unable to physically interact with FOG co-factors (*Gata^{ki}*) is only expressed in the adult small intestine after tamoxifen™ induction. No gross abnormalities could be detected in the overall structure of the intestinal epithelium of these mice, in particular we could not detect an increase in goblet cell population. However, the analysis of *LPH* and *Asbt* mRNA levels revealed that FOG co-factors facilitate in the Gata4 dependent repression of Asbt in the adult jejunum, but do not play a role in the activation of *LPH*. Therefore we conclude that the interaction of FOG co-factors, presumably Fog1, with Gata4 in the intestine is necessary for the complete Gata4 repression of ileal specific gene expression in the adult jejunum.

METHODS

Mice. Mice were housed under standard conditions in the Animal Research at Children's Hospital (ARCH) facility and provided food and water ad libitum. To generate Gata4$^{flox/ki}$ mice we crossed Gata4$^{flox/flox}$ mice (27) with Gata4$^{wt/ki}$ mice (9)(generous gift from dr. S. Orkin, Children's Hospital Boston). These mice were generated in a VillinCreER^{T2} (12) transgenic background as depicted in Fig. 4. In these mice, Cre is expressed in the epithelial cells of the small and large intestine. The expressed Cre is fused to a mutated estrogen receptor, and resides in the cytoplasm until it is translocated into the nucleus upon Tamoxifen^{TM} treatment, where it then excises floxed DNA. Treatment of *Gata4$^{flox/ki}$* Cre+ mice with tamoxifen^{TM} effectively results in a *Gata4$^{\Delta ex2/ki}$* and these mice wer designated intestinal *Gata4* knock-in mice. The phenotype of these mice are compared to the earlier described *Gata4$^{\Delta ex2/\Delta ex2}$* (mutant) mice that harbor a truncated, inactive Gata4 protein in the small intestine(3). Control mice are *Gata4$^{flox/flox}$* mice that are treated with tamoxifen^{TM}, but do not carry the VillinCreER^{T2} transgene and are therefore are phenotypically wild-type mice.

Tamoxifen treatment. Nuclear translocation of Cre was induced by daily intraperitoneal injections of Tamoxifen^{TM} (100µl, 10mg/ml) as described (12). To study Asbt expression in the *Gata4* mutant mice during weaning, mice were injected for 4 days starting at postnatal day 10 (P10) as described previously (2). The knock-in animals were all 6-8 weeks of age and were injected for 5 consecutive days, and harvested 2 weeks after the last injection, exactly as described previously (3). At the time of sacrifice, mice were anesthetized with avertin anesthesia, and the intestine was removed from a midline incision, placed on a glass plate on a bed of ice, and rinsed with ice-cold PBS. Intestinal tissue was obtained along the length of the small intestine for mRNA isolation and sectioning as described (36). Jejunum (segment 3) and distal ileum (segment 5) were the focus of this study.

Genotyping. Genotyping was conducted on genomic tail DNA to distinguish between *Gata4 wild-type*, *Gata4 floxed* and *Gata4 knock-in* alleles. Using primers directed to exon2 (F1/F2) the floxed allele could be distinguished from wild-type or knock-in alleles as described previously (3). Addition PCR-genotyping was conducted to identify the knock-in allele using primers directs towards intron 5, that holds additional sequence to mark the knock-in allele (9). To confirm effective recombination of the *floxed* alleles intestinal RNA was obtained and RT-PCR using the F1/F2 primers (Fig. 4.) was performed. Finally, the knock-in mutation was confirmed by sequencing at the Molecular Genetic Core (Children's Hospital, Boston). Jejunal cDNA obtained from *Gata4$^{\Delta ex2/ki}$* mice and a sequencing primer directed towards exon3 (N-terminal zinc finger) was used for sequencing. Primer sequences are available upon request.

RNA isolation. RNA was isolated as described previously[van Wering, 2004 #1025]. Briefly, RNA was isolated using the RNeasy kit (Qiagen) with DNase treatment, quantified by optical density at A260nm, and checked on an agarose gel for intact ribosomal RNA bands. Complementary DNA (cDNA) was synthesized using iScript (BioRad). From postnatal mice,

RNA was isolated from 30-50 mg of small intestine (0.5 to 1.0 cm) obtained from the most proximal region adjacent to the pylorus (segment 1), the 25% point (segment 2), the geometric center (segment 3), the 75% point (segment 3), and the most distal region adjacent to the ileocecal junction (segment 5). For all analyses, segments 1-5 generally correspond to duodenum, proximal jejunum, mid-jejunum, proximal ileum, and distal ileum, respectively.

Real-time RT-PCR. Real-time RT-PCR was conducted exactly as described previously (3, 4). Primer pairs were designed using Beacon Design software (Biosoft International) and optimized as described (4). Real-time RT-PCR was carried out using an iCycler and iQ SYBR Green Supermix (Bio-Rad), and all data were corrected for *Gapdh* and expressed relative to the calibrator, which was adult jejunal RNA unless indicated otherwise (indicated in figure legends). RT-PCR primer sequences are available upon request.

Immunofluoresence (IF). Immunofluoresence was performed exactly as described previously (3). Paraffin embedded intestinal segments were heated for 15 min at 65^0C, deparaffinized and rehydrated. Antigen retrieval was performed by boiling slides for 10 min in 10mM sodium citrate, pH 6.0. After cooling, slides were washed 3X for 5 min in PBS and incubated in a blocking solution containing 10% donkey serum in PBS for 1 h in a humidified chamber at room temperature. Blocking serum was replaced by the primary antibody in 10% donkey serum/PBS, and incubated overnight at 4^oC in a humidified chamber. Slides were then washed 3X for 5 min in PBS and incubated with the secondary antibody 10% donkey serum/PBS for 4 h in a humidified chamber at room temperature. In some experiments, a solution containing DAPI (4′,6-diamino-2-phenylindole dihydrochloride) nucleic acid stain (2ug/ml; D1306, Molecular Probes) in PBS was added and incubated for 15 min at room temperature. Slides were washed in PBS, mounted in Mowiol Mounting Media (Calbiochem, San Diego, CA) and allowed to dry overnight.

The primary antibodies included rabbit anti-Asbt (1:500, kind gift from Dr. P. Dawson, Wake Forest University), and rabbit anti-LPH (1:500, kind from K-Y. Yeh, Louisiana State University). The secondary antibodies used was Alexa fluor 488 donkey anti-rabbit antibody (1:500, Molecular Probes).

Plasmids. Previously characterized expression vectors for mouse Gata4 (G4-CMV)(21), and a mouse 1.1 Kb mouse Asbt-luciferase promoter-reporter construct was used as described previously (7).

Sequence analyses. The 5'-flanking region of the mouse *Asbt* gene (accession number NM_0011388) was analyzed for GATA sites using Vector NTI software (Invitrogen™).

Cell culture, transient transfections, and luciferase assays. Transient transfection in the mouse colon carcinoma cell line, CT-26, was carried out as described by Chen et. al.(7). Briefly, confluent cells were harvested and resuspended in 700 µl of PBS containing 4 µg of mouse *Asbt*-luciferase reporter constructs and 0.1 µg of a control plasmid (pRL-TK) encoding the *Renilla* luciferase gene under the control of the thymidine kinase promoter (Promega). The cells were transfected by electroporation. Luciferase activities were determined by the dual luciferase

reporter assay system (Promega) according to manufacturer's instructions, using a Turner 20/20^{n} Luminometer (Turner BioSystems) with a 10-s counting window.

In situ hybridization (ISH). The ISH experiments shown in this chapter are performed in the laboratory of dr. S. Orkin, Department of Hematology and Oncology, Children's Hospital Boston and shown with permission.

RESULTS

Previously we found that ileal genes, such as the apical sodium bile-acid transporter (*Asbt*), are induced in the jejunum of adult mice in which the activation domains of Gata4 are deleted (3), implicating Gata4 as an important regulator of the ileal restricted expression of Asbt in adult mice. Asbt expression however is not only under tight horizontal control, it also demonstrates an evolutionarily conserved developmental expression profile. In humans, dogs, rats, rabbits and guinea pigs an up regulation of *Asbt* during postnatal development in the ileum has been described(1, 11, 16, 22, 23, 26, 28), however the underlying mechanism for this temporal expression remains to be elucidated. Recently we showed efficient intestinal specific Gata4 inactivation during weaning by tamoxifen™ treatment of the inducible, intestinal *Gata4* mutant mice between postnatal day 7 (P7) and P10 (2). Making use of this Gata4 inactivation model we now sought to define the role of Gata4 in the horizontal *Asbt* gene expression during weaning by quantifying *Asbt* mRNA levels with real-time RT-PCR, and protein expression by immunofluoresence in control and *Gata4* mutant mice (Fig. 1).

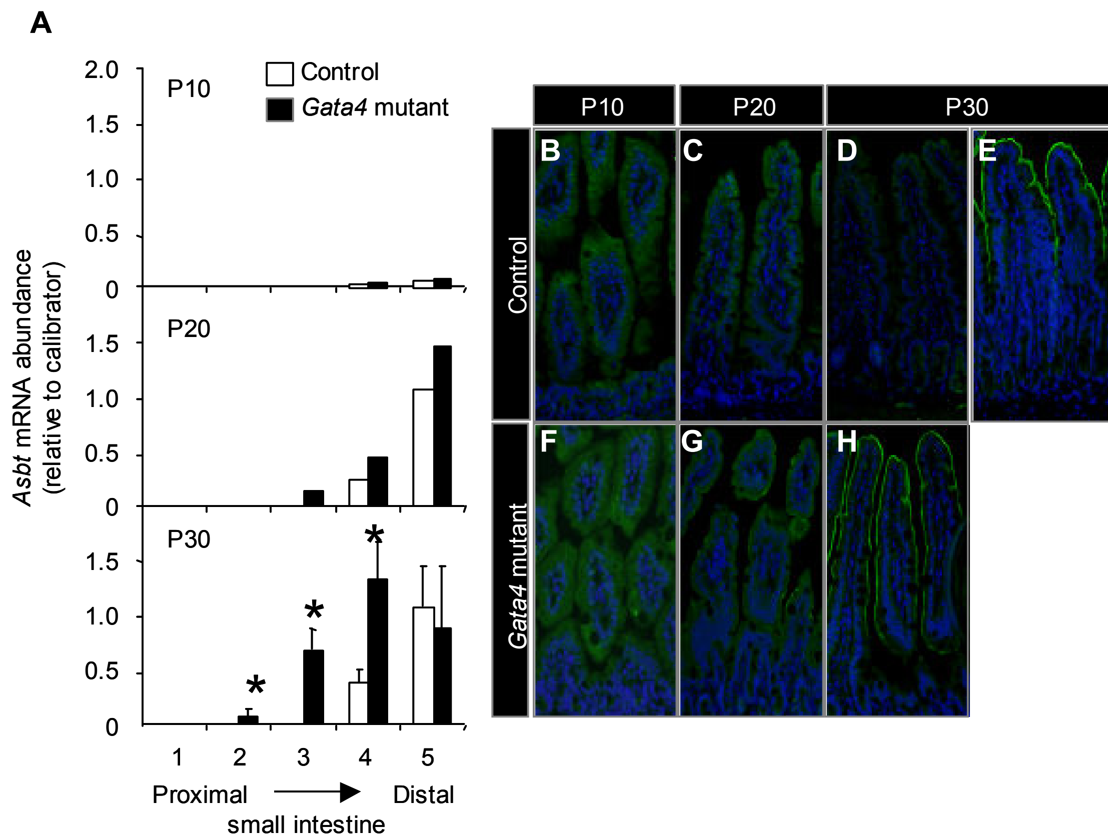

Fig. 1. Effects on Asbt expression after Gata4 inactivation during weaning. *Asbt* mRNA and protein expression was determined in control and *Gata4* mutant mice at postnatal day 10 (P10, preweaning), P20 (weaning), P30 (postweaning). (A) Real-time RT-PCR analysis of *Asbt* mRNA levels at P10 (top, N=4), P20 (middle, N=2) and P30 (bottom, N=7) along the length of the small intestine in control (open bars) and Gata4 mutant mice (filled bars). The x-axis indicates the position along the length of the small intestine (1=proximal duodenum, 2=proximal jejunum, 3=mid-jejunum, 4=proximal ileum, 5=distal ileum) and all data are controlled by *Gapdh* and expressed relative to a calibrator (pool of mRNA obtained from control adult ileum;seg5). (B-D) Immunofluoresence using an Asbt-antibody showed the lack of Asbt expression in the mid-jejunum (seg3) sections of P10, P20, P30 control mice, and (E)abundant Asbt expression (green) in the ileum of a P30 control section. (F-H) Asbt immunostaining of mutant jejunal sections at P10, P20 and P30, showed matching with the mRNA data the jejunal expression of Asbt in the P20 and P30. Slides were co-stained with DAPI nuclear stain (blue) indicating the nuclei.

Preweaning, at P10, both control and Gata4 mutant showed nearly undetectable levels of *Asbt* mRNA throughout the small intestine (Fig.1A), indicating that Gata4 does not play a critical role in the repression of mouse *Asbt* expression at this time. During weaning, endogenous *Asbt* mRNA expression (as indicated by the open bars) increased significantly between P10 and P20 and is limited to the ileum (segment 4 and 5) of the mouse small intestine, parallel to the developmental expression profile described in rats (29). In the *Gata4* mutant mice (filled bars) we detected an increase of *Asbt* mRNA expression during weaning similar to the control group, however in contrast to the control mice the increase was not limited to segment 4 and 5 but

extended into segment 3 of the P20 mutant mice. This finding in the P20 mutant mice is consistent with the role of Gata4 as a repressor of *Asbt* gene expression in the small intestine. The horizontal distribution of Asbt mRNA after weaning (P30) in control animals showed a pattern similar to that of the P20 mice, with high levels in the ileum (segment 4 and 5) and undetectable low levels in the rest of the small intestine. The *Asbt* mRNA abundance in the ileal segments (4 and 5) of the *Gata4* mutant mice at P30 was not significantly different from control mice. However the induction of *Asbt* mRNA in the mid-jejunum (segment 3) at P20 had increased further in the P30 mutant mice reaching near ileal levels (N=7, P<0.05). In addition in the P30 mutant mice, *Asbt* mRNA was also detectable in the proximal jejunum (segment 2), indicating that the inactivation of Gata4 has resulted in a distal shift of ileal gene expression. To investigate if the Asbt protein follows the *Asbt* mRNA levels immunofluoresence experiments were conducted (Fig. 1B-H). In the P30 control ileum (Fig. 1E), Asbt staining was specific to the microvillus membrane consistent with normal high mRNA levels. Asbt protein was absent in the jejunum of control mice at all developmental time-points (Fig. 1B-D), matching the mRNA results. Conversely, but consistent with the mRNA data, Asbt protein was detectable on some enterocytes in the jejunum of the *Gata4* mutant mice at P20 (Fig. 1G). Furthermore, section of the jejunum of *Gata4* mutant mice at P30 (Fig. 1H), strongly expressed Asbt protein on the brushborder, similar to the control ileum. Together these data are consistent with the results obtained in adult mice, identifying Gata4 as a potent repressor of Asbt expression in the jejunum. These data further show that Gata4 is a critical factor in determining the distal boundary of *Asbt* expression along the horizontal axis established during weaning in mice.

Although the repressor function of Gata4 has now been identified, the underlying mechanism of Asbt repression remains to be determined. We have shown previously that other potential regulators of Asbt, including *Hnf1α*, *c-Jun*, *c-Fos* and *liver receptor homologue-1* (*lrh1*) are not effected by the inactivation of Gata4, providing evidence that the repression is a direct effect of Gata4 on the promoter of *Asbt*. Keeping with this hypothesis, we searched the mouse Asbt 5'-flanking region (*NM_0011388*) for putative Gata4 binding sites (Fig2A). This analysis revealed the presence of 6 GATA binding sites in the first 1.1 kb Asbt promoter, of which 2 conserved sites in the first 300 bp downstream of the transcriptional start site (conserved in human, rat and rabbit). To determine if the Gata4 dependent repression acts through the 1.1 kb mouse Asbt promoter, we used this promoter fused to a luciferase reporter in transient co-transfection assay using CT-26 cells (Fig. 2B).

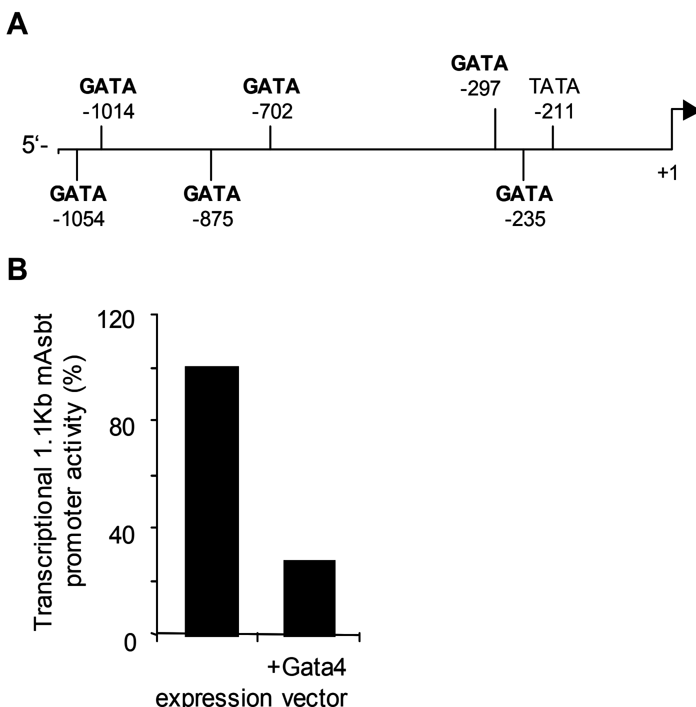

Fig. 2. The mouse 1.1 kb Asbt promoter sequence contains the information for Gata4 specific repression. (A) Sequence analysis of the mouse 1.1 Kb Asbt 5'-flanking region revealed the presence of 6 putative GATA sites. (B) Transient co-transfections assay was carried out in CT-26 cells using a 1.1 Kb mouse Asbt-luciferase promoter-reporter construct transfected singly and together with a wild-type expression vector for Gata4. The data are presented as the luciferase activity relative to the luciferase activity of the Asbt promoter transfected singly. (N=2)

The CT-26 cell line is a cell line isolated from a mouse adenocacrinoma of the colon, and was used since these cells display ileal-like characteristics (5). When we transfect the *Asbt*-luciferase promoter-reporter construct in these cells, they produce high luciferase levels indicating that the transcriptional machinery to activate the *Asbt* promoter is present in the CT-26 cells. We next co-transfected the *Asbt* promoter construct together with a wild-type Gata4 expression vector. This resulted in a 70% reduction of luciferase activity compared to transfection of the *Asbt* promoter constructs without Gata4. Together these results suggest that the Gata4 dependent repression is regulated through the first 1.1 kb mouse *Asbt* promoter and show that the CT-26 cell line is a adequate *in vitro* model system to further investigate the underlying mechanisms.

Since Gata4 is generally considered an activator we hypothesized that the observed ability to repress ileal genes may be the result of specific interaction with other factors that modulate Gata4 function. One family of regulators that is emerging as a family that modulates GATA function in many organ systems is the friend of GATA (FOG) family of co-factors. To mediate GATA function, FOG co-factors are mostly co-expressed with GATA factors during mouse development in a variety of organ systems (31, 32, 35). Despite many reports describing the expression of Gata4, 5 and 6 in the adult mouse intestine, the expression of FOG co-factors in the adult small intestine has not yet been reported. To provide a topographical basis for a functional interaction with Gata4 in the adult intestine, we determined the expression patterns of *Fog1* and *Fog2* in the adult gastrointestinal system using in situ hybridization. Sections of adult

stomach, duodenum, jejunum and ileum, cecum and colon of wild-type mice were hybridized with a probe specific for *Fog1* and *Fog2* mRNA (Fig. 3A and B).

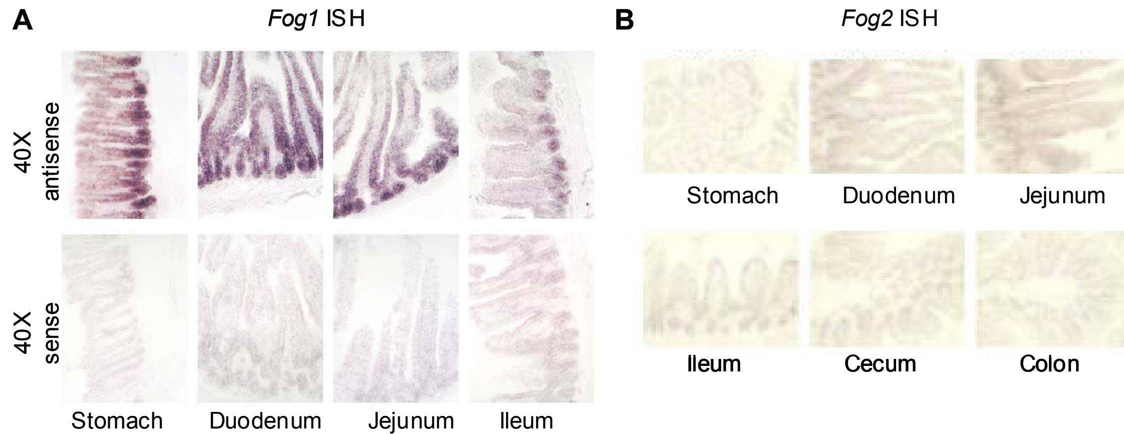

Fig. 3. Expression of FOG co-factors in the gastrointestinal system. *Fog1* and *fog2* mRNAs are expressed in the epithelium of adult mouse gastrointestinal tract. (A) In situ hybridization using a probe specific for Fog1 showed high levels of Fog1 in stomach, duodenum, jejunum and ileum. The anti-sense probe was used as a negative control. (B) In situ hybridization using a probe for *Fog2* revealed low levels of *Fog2* expression in duodenum, jejunum and ileum and no expression in cecum and colon. These experiments were conducted in the laboratory of our collaborator, Dr. S. Orkin (Children's Hospital Boston) and shown here with permission.

We found expression of both *Fog1* and *Fog2* in the mouse stomach epithelium and along the length of the small intestinal epithelium with signals being stronger in crypts and at the base of villi than at the villus tips. In situ signals for *Fog1* mRNA were more intense in duodenum than ileum, and overall, Fog1 signals were much stronger than *Fog2* signals, implicating Fog1 as the principal FOG co-factor in the small intestinal epithelium. The horizontal pattern of *Fog1* mRNA expression (distal decline) parallels the expression of Gata4 in the adult intestine, suggestive for associate functions.

The investigate the role of a potential interaction between FOG co-factors and Gata4 in the adult small intestine, we overcame the early lethal phenotype of homozygous *Gata4* knock-in mice (9) by establishing an inducible, intestine-specific *Gata4* knock-in model (Fig. 4). In this model, homozygous floxed *Gata4* ($Gata4^{flox/flox}$) mice (27) are crossed with heterozygous *Gata4* knock-in ($Gata4^{wt/ki}$) mice to generate mice heterozygous for the floxed *Gata4* and the knock-in *Gata4* allele ($Gata4^{flox/ki}$). The knock-in mutation results in a previously validated mutant Gata4 protein with a valine to glycine substitution on position 217 (V217G) in the N-terminal zinc finger, that specifically disrupts the binding of FOG co-factors (9). To enable us to induce

intestinal specific recombination these mice were also bred with a transgenic line (Villin-CreER^{T2}) in which Cre is expressed in the epithelial cells of the small and large intestine under the control of the villin promoter (12). The expressed Cre is fused to a mutated estrogen receptor, and resides in the cytoplasm until it is translocated into the nucleus upon Tamoxifen™ treatment, where it then excises floxed DNA. As previously shown for the *Gata4$^{flox/flox}$* mice (3), upon Tamoxifen™ treatment the recombination of exon2 is intestinal specific and results in the *Gata4$^{\Delta ex2}$* allele.

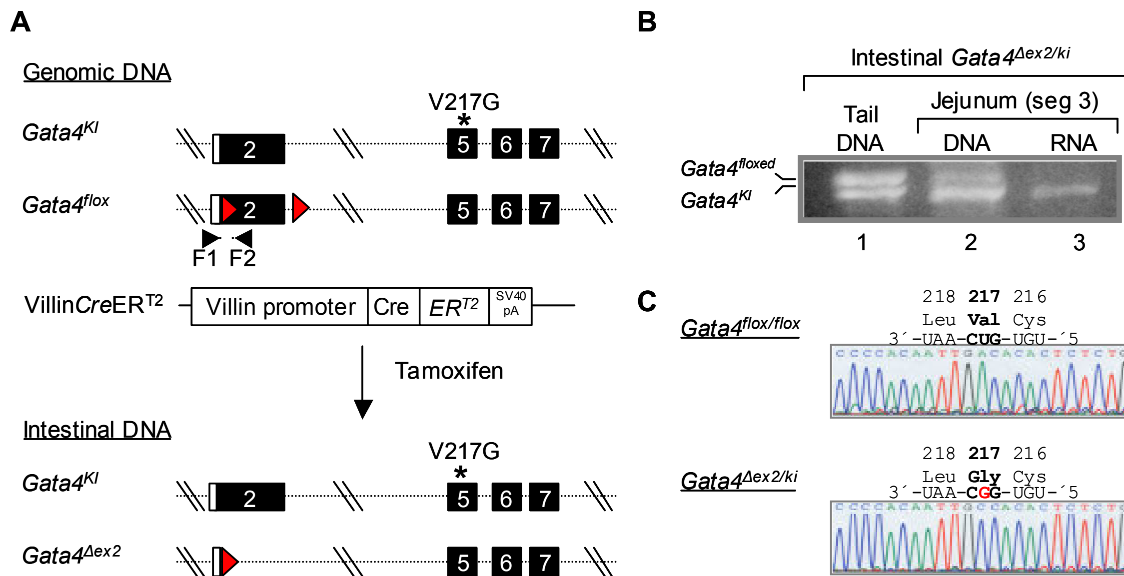

Fig. 4. VillinCreER^{T2} mediated recombination of the *Gata4$^{floxed/ki}$* allele in the mouse small intestine results in intestinal, inducible Gata4 knock-in mice (*Gata4 $^{\Delta ex2/\ \Delta ex2}$*). (A) Schematic representation of the Gata4 and VillinCreER^{T2} alleles used. The *Gata4^{flox}* allele has 2 loxP sites (indicated by the red arrowheads) flanking the 2nd exon of Gata4. The *Gata4^{ki}* allele has a mutation in exon5 that results in a valine to glycine substitution on position 217 (V217G). *Gata4$^{\Delta ex2}$* allele represents the Gata4 locus after intestinal specific Cre mediated recombination. Arrows indicate location of primers used for genotyping. (B) Genotyping of the Gata4 $^{\Delta ex2/ki}$ using the F1 and F2 primers. In lane 1, PCR with tail DNA of these mice shows the presence of both the floxed (top band) and the knock-in (bottom band) alleles in genomic DNA. Intestinal DNA (lane 2) showed that the floxed allele is recombined (remaining signal is the result of contamination of the non-epithelial fraction) and the knock-in allele is unaffected. RT-PCR with jejunal RNA (lane 3) shows efficient recombination of the floxed allele (absence of top band) and the presence of the Gata4 knock-in allele. (C) Sequence analysis of exon5 of jejunal RNA from control (*Gata4$^{flox/flox}$*) and the intestinal knock-in (*Gata4 $^{\Delta ex2/ki}$*) mice confirmed the presence of the knock-in mutation that results in a valine to glycine substitution on position 217 in the N-terminal zinc finger of Gata4.

This allele synthesizes a transcriptionally inactive Gata4 mutant protein that lacks the Gata4 activation domains as described previously(3). In the current experiment, mice harboring the VillinCreER^{T2} transgene and *Gata4$^{flox/ki}$* were treated with Tamoxifen™ for 5 consecutive days resulting in *Gata4$^{\Delta ex2/ki}$*, and tissue was harvested 2 weeks after the last injection. The genotype of these mice was confirmed by PCR using tail DNA and primers flanking the 3'-LoxP site as shown in figure 4 (lane 1). Intestinal specific recombination was verified by (RT-)PCR on intestinal DNA and cDNA, and showed the specific deletion of exon2 in the floxed allele (lane 2 and 3). To confirm the presence of the knock-in mutation (V217G) we sequenced exon3 of jejunal RNA from the *Gata4$^{\Delta ex2/ki}$* mice and compared it with the sequence of jejunal RNA from *Gata4$^{flox/flox}$* (Fig. 4C), this showed the presence of a T to G substitution resulting in a *valine* to *glycine* substitution at position 217 of Gata4. Together these data demonstrate the successful generation of mice that upon tamoxifen™ treatment express two mutant Gata4 proteins specifically in the small intestine. One inactive truncated form (Gata4$^{\Delta ex2}$), and one Gata4 mutant that lost the ability to interact with FOG co-factors (Gata4^{ki}) effectively resulting in an inducible, intestinal *Gata4* knock-in mouse model.

The overall structure of the jejunum of the *Gata4$^{\Delta ex2/ki}$* mice did not show gross abnormalities compared to control mice (*Gata4$^{flox/flox}$*) (Fig.5).

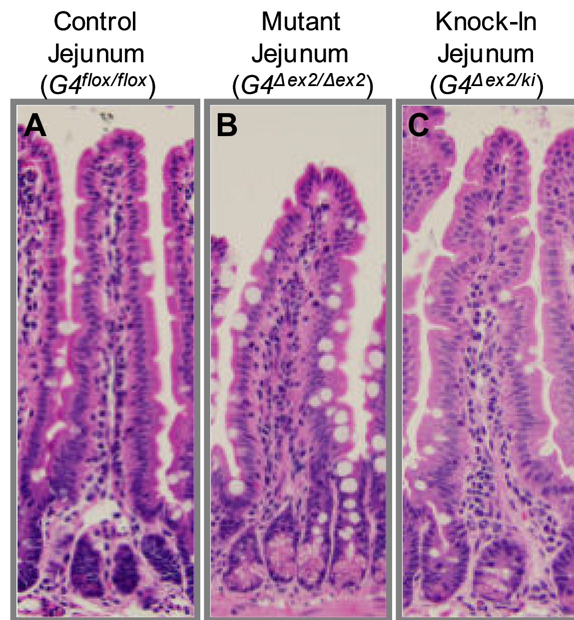

Fig. 5. No gross abnormalities in epithelium morphology of the intestinal *Gata4* knock-in mice. Hematoxilin and eosin (H&E) staining of (A) control jejunum (Gata4$^{flox/flox}$), (B) mutant jejunum (*Gata4$^{\Delta ex2/\Delta ex2}$*) and (C) the jejunum of *Gata4* knock-in mice (*Gata4$^{\Delta ex2/ki}$*) revealed no gross abnormalities in the Gata4 knock-in mice.

Because we previously showed that the deletion of the activation domains of Gata4 in the intestine results in the increase in goblet cell number, we specifically explored the number of

goblet cells in the jejunum of the *Gata4*$^{\Delta ex2/ki}$ mice. Although, we could confirm the increase in goblet cells in the jejunum of age-matched *Gata4*$^{\Delta ex2/\Delta ex2}$ mutant mice (Fig. 5B), we did not see an increase in goblet cells in the jejunum of the intestinal *Gata4* knock-in mice (Fig. 5C) as compared to control mice (Fig 5A). These results suggest that the ability of Gata4 to repress the goblet cell number in the adult jejunum is mediated through the N-terminal activation domains of Gata4 and does not require the interaction with FOG co-factors.

To determine if the interaction of FOG co-factors with Gata4 in the intestine plays a role in the Gata4 dependent gene expression in the adult jejunum, we compared the expression of LPH and Asbt in the control (*Gata4*$^{flox/flox}$), mutant (*Gata4*$^{\Delta ex2/\Delta ex2}$) and knock-in jejunum(*Gata4*$^{\Delta ex2/ki}$) and control ileum using real-time RT-PCR and immunofluoresence (Fig. 6.). The deletion of the activation domains of Gata4 in the jejunum as in the Gata4 mutant mice resulted in a strong reduction of LPH mRNA levels (Fig 6A), towards ileal levels consistent with our previous report (3). The LPH mRNA levels in the jejunum of the Gata4 knock-in mice, however, were not significantly different from the levels in the jejunum of control mice. The LPH protein levels in these mice followed the mRNA results as shown by immunofluoresence (data not shown). These indicate that the disruption of the interaction between FOG co-factors and Gata4 in the adult jejunum has no effect on LPH expression. Therefore these data show that the role of Gata4 as an critical activator of jejunal genes (such as LPH) is independent of FOG co-factors.

We next determined the role of FOG co-factors in the Gata4 dependent repression of the ileal specific gene Asbt in the jejunum of adult mice (Fig. 6B and C). The *Asbt* mRNA levels in the jejunum of the *Gata4* mutant mice were induced compared to undetectable low levels in the control jejunum, confirming our previous data (3). Interestingly, in the jejunum of the *Gata4* knock-in mice we also detected an increase in *Asbt* expression compared to the control jejunum. This increase in *Asbt* expression was also confirmed by immunofluoresence, in which positive Asbt immunostaining could be detected in the jejunal sections of both *Gata4* mutant and *Gata4* knock-in mice (Fig. 6C). Together these data implicate that the Gata4 dependent repression of Asbt expression in the adult jejunum is partly dependent on the interaction of FOG co-factors with the N-terminal zinc finger of Gata4.

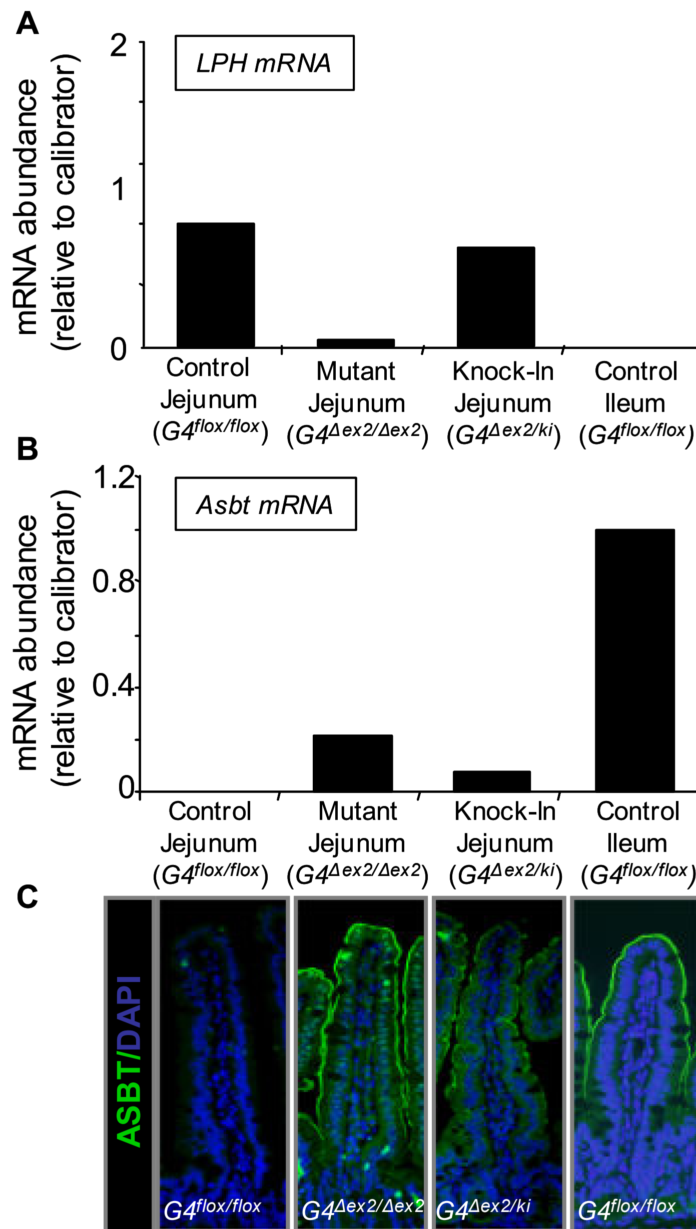

Fig. 6. The role of FOG co-factors in Gata4 dependent gene expression in adult jejunum. (A) LPH mRNA levels in control jejunum, Gata4 mutant jejunum, Gata4 knock-in jejunum and control ileum as determined by real-time RT-PCR (N=1). LPH mRNA levels are controlled for Gapdh and expressed relative to a calibrator (pool of mRNA from control jejunum, seg3). (B) Asbt mRNA levels in control jejunum, Gata4 mutant jejunum, Gata4 knock-in jejunum and control ileum as determined by real-time RT-PCR (N=1). Asbt mRNA levels are controlled for Gapdh and expressed relative to a calibrator (pool of mRNA from control ileum, seg5). (C) Asbt immunofluoresence (green) on section of a control jejunum, Gata4 mutant jejunum, Gata4 knock-in jejunum and control ileum. Nuclei are co-stained with DAPI nuclear stain.

DISCUSSION

The precisely coordinated spatial and temporal control of intestinal gene expression necessary for the maintenance of intestinal differentiation is likely the result of the interplay of multiple regulatory mechanisms. Gata4 is emerging as a major positional factor involved in establishing horizontal gene expression boundaries in the adult small intestine. In this report we defined the role of Gata4 and FOG co-factors in the determination of the proximal boundary of *Asbt* gene expression in the mouse small intestine. We show that in mice where Gata4 is inactivated before weaning the Asbt expression after weaning extends into the proximal jejunum, whereas in control animals Asbt gene expression is restricted to the ileum. We also show that the repression of Asbt can be recapitulated in an in vitro model system, using CT-26 cells and 1.1kb mouse Asbt promoter, indicating that the Gata4 repression acts through this 1.1kb promoter. In addition we identified Fog1 as the principal intestinal FOG co-factor, as suggested by the strong *in situ* hybridization signal compared to very low *Fog2* levels. The expression of *Fog1* together with Gata4 in the intestine provides a topographic basis for function interaction. The function of the assumed interaction between intestinal FOG co-factors and Gata4 was tested by the establishment of an intestinal specific *Gata4* knock-in mouse in which a Gata4 protein is expressed that can not interact with FOG co-factors. This revealed that the role of Gata4 as an activator of jejunal genes and a repressor of goblet cell number in the adult jejunum is independent of FOG co-factors. However, these mice also revealed that the Asbt repression by Gata4 is partly dependent on the interaction with intestinal FOG co-factors, presumably Fog1.

Given the overall importance of Asbt function in health and human disease, precise understanding of the molecular mechanisms of its regulation is of great importance. Exploitation of endogenous mechanisms of the jejunal down-regulation of this gene will provide novel approaches to the treatment of patient with lost ileal function, such as in inflammatory bowel disease. The experiments described in this manuscript identify intestinal Gata4 and FOG co-factors as key factors involved in directing the proximal boundary of Asbt expression. The identification of the mechanism of Gata4 dependent Asbt repression, may lead to new ways to treat patients with short guts, where ileal function has irreversibly been lost. Similarly, identification of factors involved in the developmental regulation of Asbt expression will lead to new methods to care for premature infants, where intestinal function is immature.

Functional GATA:FOG interactions have been characterized in other tissues, including bone marrow, testes, heart and stomach. Cooperation between Gata1 and Fog1 is required for normal erythropoiesis (8) and within the megakaryocyte lineage Fog1 acts with either Gata1 or Gata2 to direct differentiation of early precursors(6, 14) (Nichols, Chang, Cantor, Garriga-Canut and Orkin). Fog1 is co-expressed with Gata3 in naïve T-cells and may repress T-helper 2 cell development (37). Interactions between Gata4 and Fog2 are required for early testis development and stomach regionalization. Normal cardiogenesis requires interactions between members of the Gata4, 5, and 6 subfamily and both Fog1 and Fog2 (9, 20). In this study we show consistent with

these studies in other organ systems, that also in the small intestine FOG co-factors play a role in GATA function. Specifically we show that the Gata4 dependent repression of the ileal gene, Asbt, in the adult jejunum is partly dependent upon interactions with FOG family members, presumably Fog1.

In the gastro-intestinal system Gata4 is emerging as a key factor in establishing horizontal boundaries of gene expression. Previously the role of Gata4 in stomach differentiation and development has been described (19), using mosaic *Gata4* null mice. This report first shows that Gata4 is specifically expressed in the epithelium of the hind-stomach and not in the fore-stomach. The patches of Gata4 null cells failed to properly differentiate, and these patches expressed high Shh levels in the hind-stomach whereas endogenously Shh expression is restricted to the fore-stomach. This study established Gata4 as a critical regulator in the differentiation and regional specification of the stomach epithelium in mice. Recently another report analyzed the expression of FOG co-factors in the stomach epithelium and found that Fog1 is the predominant FOG factor in the stomach. In this report, *Gata4* knock-in embryos were used to show that they also had increased levels of Shh in the hind-stomach, indicating that the role of Gata4 in specifying the regional diversity between fore- and hind-stomach is dependent on interaction with FOG-cofactors. Our results in the adult small intestine as describe in this report show similarities with the gastric literature. We demonstrate that Gata4:FOG interactions play a role in maintaining the jejunal-ileal differences by repressing the ileal gene, Asbt, in the jejunum of adult mice. The analyses of the phenotypes of the knock-in mice described, has been biased towards hypothesized target genes based on previous results. Collectively these studies therefore implicate the interplay of Gata4 with FOG co-factors (likely Fog1) in the gastro-intestinal epithelium as a mechanism to establish and maintain regional diversity.

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CHAPTER 7

Summary and discussion of future perspectives

Tjalling Bosse[1], Richard J. Grand[2], Hans A. Büller[3] and Stephen D. Krasinski[2]

From the [1]University of Amsterdam, Department of Pediatrics, Amsterdam Medical Center, Amsterdam, 1100DD; The Netherlands; [2]Division of Gastroenterology and Nutrition, Department of Pediatrics, Childrens Hospital Boston, Boston MA, 02115; Department of Pediatrics, Harvard Medical School;[3]Erasmus University Rotterdam, Department of Pediatrics, Erasmus MC/Sophia Children's hospital, Rotterdam, The Netherlands

7. Summary and discussion of future perspective

7.1 Summary

This thesis describes the role of transcription factors Gata4 and Hnf1α in the regulation of intestinal gene expression in mice, using lactase-phlorizin hydrolase (*LPH)* and sucrase isomaltase (*SI)* as the principal marker genes for intestinal differentiation and development. **Chapter 1** is a general review on the current understanding of intestinal differentiation and development and describes in detail previous work from the laboratory of R.J. Grand and S.D. Krasinski as well as work from other laboratories with respect to the complex mechanisms regulating intestinal gene expression. Experiments are discussed that established *LPH* and *SI* as excellent markers for intestinal differentiation and show that expression of these genes is regulated on the level of gene transcription. LPH and SI are both brush border hydrolases of the absorptive enterocyte, which are responsible for the digestion of lactose (the principal carbohydrate of mammalian milk) and α-disaccharides (mainly found in solid foods) respectively. During intestinal development, critical transitions are marked by the changes in *LPH* and *SI* expression facilitating the changes in dietary content. Earlier work showed a close correlation between transcriptional rate of the *LPH* and *SI* gene with the protein activity, protein, mRNA and pre-mRNA along the length of the intestine and during development. In addition transgenic studies indicated that information for the expression patterns of *LPH* and *SI* along the horizontal, vertical and temporal axes are under intrinsic control of the 5′-flanking regions. Together these studies have established that *LPH* and *SI* biosynthesis is regulated predominantly by transcriptional regulation. DNA sequence analysis of the 5′-flanking regions (proximal promoters) of *LPH* and *SI* revealed the presence of evolutionarily conserved *cis*-elements in close proximity to each other and the TATA-box. Among these elements, binding sites for the zinc finger containing GATA family and the homeodomain containing HNF-1 and Cdx families were found. Recent studies by us showed that GATA and HNF-1 factors are capable of physically interacting with each other and that this association is required to achieve functional synergy of the *LPH* and *SI* promoters *in vitro*. This fundamental finding by dr. Herbert M. van Wering has led to the central paradigm of this thesis, that the overlapping expression of these two conserved transcription factor families is necessary for intestine-specific expression of certain target genes *in vivo* and consequently may contribute to intestinal differentiation and development. This thesis reveals the role of Gata4 and Hnf1α in intestinal specific gene expression, by using mouse knock-out models, and represents an advance in our understanding of the mechanisms controlling gut differentiation, development and function.

7.1.1 Gata4 is a key regulator of LPH gene expression

In **chapter 2** it is revealed that Gata4 is the principal intestinal GATA factor that binds to the GATA binding site on the mouse *LPH* promoter and its expression is highly correlated with that of LPH in the jejunum and ileum. To delineate function, the relationship between *LPH* mRNA

and Gata4 protein was determined along the length of the mouse small intestine using quantitative RNase protection assays (RPA), western blots and qualitative immunohistochemistry (IHC). Intestinal Hnf1α expression patterns were also determined, because we have previously shown that Gata4 cooperatively activates intestinal genes together with Hnf1α. Whereas Hnf1α was found to be expressed equally throughout the small intestine, Gata4 was nearly undetectable in the distal ileum, consistent with low levels of *LPH* mRNA in this region. Collectively these data indicate for the first time that Gata4 is a primary GATA regulator of *LPH* gene expression in the adult small intestine.

Chapter 2 also describes the mechanism of *LPH* promoter activation by Gata4 and Hnf1α together (similar to the mechanism described earlier for Gata5) and shows that Gata4 is able to activate the *LPH* promoter independent of Hnf1α, contrasting with Gata5. To determine these mechanisms of Gata4 activation we used HeLa cells, because these cells do not synthesize endogenous Hnf1α or Gata4. We show that the mechanism for functional synergy between Gata4 and Hnf1α to activate the *LPH* promoter required physical association between Gata4 and Hnf1α (GST pull-down assays), DNA binding (EMSAs) and intact HNF-1 binding sites on the *LPH* promoter (mutagenesis in transient co-transfection assays). The mechanism of Hnf1α-independent *LPH* activation by Gata4 required intact GATA binding sites on the *LPH* promoter and intact Gata4 activation domains. Elegant structure-function studies, in which zinc fingers and basic region of Gata4 and Gata5 are interchanged, revealed that the ability of Gata4 to independently activate the *LPH* promoter is mediated by the zinc fingers and basic region of Gata4. EMSA binding studies, however, showed that the difference in the capacity of Gata4 and Gata5 to activate the *LPH* promoter was not due to a difference in affinity for binding to the GATA binding sites on the *LPH* promoter. These data indicate that Gata4 is a key regulator of *LPH* gene expression that may function through an evolutionarily conserved mechanism involving cooperativity with Hnf1α and/or a Gata4-specific pathway that is independent of Hnf1α.

7.1.2 Hnf1α regulates a subset of intestinal genes in the adult jejunum

In **chapter 3** we moved away from cell culture models dependent on the over-expression of transcription factors and extra-chromosomal promoter reporter plasmids, by using *Hnf1α* null mice to characterize the role of Hnf1α in the regulation of intestinal gene expression *in vivo*. In this chapter *LPH* and *SI* gene expression levels as well as levels of other putative Hnf1α targets were analyzed in adult *Hnf1α* null mice relative to wild-type littermates. To quantify the mRNA abundance of *LPH* and *SI,* both RPAs and real-time RT-PCR were conducted on intestinal tissue sections. We found that in adult mouse jejunum, *LPH* mRNA in *Hnf1α* null mice was reduced 95% as compared to wild-type controls ($P<0.01$, $n=4$), showing for the first time the essential role of Hnf1α in the regulation of *LPH* gene expression in adult mouse intestine. Surprisingly, *SI* mRNA abundance was unchanged in the absence of Hnf1α along the length of the adult mouse small intestine as well as in newborn jejunum. This was an unanticipated finding and showed,

despite an abundance of literature that suggests Hnf1α as a regulator of *SI* gene expression, that Hnf1α is dispensable for *SI* gene expression *in vivo*. Together this manuscript was the first to show *in vivo* Hnf1α-requirement for *LPH* gene expression and a surprising lack of Hnf1α-requirement for *SI* gene expression.

Further in **chapter 3**, we have developed chromatin immunoprecipitation assays (ChIP) on mouse epithelial cells. This assay has provided us with a tool to study protein-DNA interactions and histone modifications in an *in vivo* context. Using ChIP assays, we show for the first time that Hnf1α occupies the proximal promoters of both the *LPH* and *SI* genes *in vivo*. These data show that the *in vivo* Hnf1α-DNA interaction, such as that on the *SI* promoter, cannot directly be translated in an Hnf1α requirement for transcriptional activity. We also could not identify a relationship between Hnf1α requirement and promoter acetylation state. We found that in wild-type and in the *hnf1α*$^{-/-}$ mouse jejunum the *LPH* and *SI* promoters were hyperacetylated, indicating that there is no relation between the histone acetylation state and the requirement for Hnf1α in the adult mouse jejunum. Finally using semi-quantitative RT-PCR, a set of hypothesized intestinal target genes was tested for their expression in *Hnf1α* null mice, both in adults and in newborn jejunums. These experiments showed that only a subset was regulated by Hnf1α *in vivo*, and this regulation occurs in a defined developmental context. Together the data in this chapter indicate that Hnf1α is an activator of a subset of intestinal genes (including *LPH*, but not *SI*), and induces these genes through a mechanism other than chromatin remodeling.

7.1.3 Gata4 is necessary for the maintenance of jejunal-ileal identities in the adult small intestine

In **chapter 4** we determined the role of Gata4 in the adult mouse intestine making use of a newly established conditional knock-out model. Conflicting data in the literature has prompted us to begin this analysis by defining the cellular distribution of Gata4 in adult wild-type jejunum (using co-IF). Consistent with previous data we show that Gata4 is highly expressed in the absorptive enterocytes on the villus, however we also note Gata4 expression in the proliferative compartment as shown by co-staining with Ki67 (a marker for proliferating cells). Low levels of Gata4 expression were also found in the Paneth cells, but Gata4 was distinctly not expressed in the goblet and enteroendocrine cell lineage. Gata4 was further found to be co-localized with Gata6 in the absorptive enterocytes of the adult mouse jejunum. Additionally, absence of Gata4 protein in the distal ileum was shown to be conserved in humans. The conserved lack of expression in the distal gut was the basis for the hypothesis that Gata4 may be functioning as a positional factor involved in maintaining differences between jejunum and ileum. To test this hypothesis *in vivo* it was necessary to inactivate Gata4 in the intestine in adult mice and therefore an intestine specific, inducible Gata4 knock-out mouse line was established. In this model, a previously validated mouse line was used in which a portion of exon 2 containing the translational start site and N-terminal activation domains of Gata4 was flanked by loxP sites (floxed). *Gata4*$^{flox/flox}$ mice were mated with an established transgenic line (Villin*Cre*ER^{T2}) in

which *Cre* is expressed in the epithelial cells of the small and large intestine under the control of the villin promoter. The expressed *Cre* is fused to a mutated estrogen receptor, and resides in the cytoplasm until it is translocated into the nucleus upon Tamoxifen^{TM} treatment, where it then excises floxed DNA. The treatment of *Gata4*$^{flox/flox}$; VillinCreER^{T2} positive mice, resulted in the inactivation of Gata4 in the adult jejunum.

The phenotype of the treated *Gata4* mutant mice revealed novel Gata4 functions consistent with the hypothesis that Gata4 may act as a positional factor in the adult gut. The jejunal epithelium of the *Gata4* mutant mice showed signs of ileal transformation based on cell composition and gene expression. Previously identified Gata4 targets normally highly expressed in the jejunum, such as *LPH* and *Fabp1*, were attenuated in the mutant Gata4 mice as shown by IF and real-time RT-PCR (10% and 20% of wild-type expression, respectively). In contrast, *SI* gene expression in the jejunum of the mutant animals was unaffected corresponding to the findings in the *Hnf1α* null mice; however, contrasting with the cell culture literature. Surprisingly, the absorptive enterocytes of the mutant Gata4 jejunum expressed high levels of the ileal specific genes *Asbt* and *Ilbp* as shown again by IF and real-time RT-PCR (20 and 644 fold increase, respectively). The altered phenotype was not restricted to the absorptive enterocytes, but also involved the goblet and enteroendocrine populations. Consistent with the ileal-shift, the goblet cell number was increased and the composition of the subpopulations of enteroendocrine cells were ileal like in the jejunum of the mutant *Gata4* mice. This shift in cell-fate reveals a novel function of Gata4 that may explain the presence of Gata4 in the early proliferating cells. To further explain the cell-fate effect, we analyzed components of the Wnt and Notch pathway and found a significant increase in *Math1* gene expression, but no difference in other components of the Notch or Wnt pathways. Since Math1 is responsible for the formation of the secretory lineage in the gut epithelium, the increase in *Math1* is consistent with the observed increase in goblet cell populations. Taken together, making use of state of the art knock-out technology, this chapter reveals novel unexpected and physiologically important functions of Gata4 in the small intestine. It can be concluded that Gata4 is a major and essential positional factor maintaining differences between jejunum and ileum in the adult mouse small intestine. These novel findings are central to this thesis and are the basis of many future studies that will be pursued (**see 7.2 Discussion of future perspectives**).

7.1.4 The role of Gata4 and Hnf1α in the regulation of gene expression during intestinal differentiation and development

In **chapter 5** we present experiments that define the role of Hnf1α and Gata4 transcription factors in the initiation and maintenance of *LPH, Fabp1* and *SI* gene expression during intestinal cytodifferentiation, suckling and weaning *in vivo*. The results of inactivating Gata4 or the absence of Hnf1α on *LPH*, *Fabp1* and *SI* gene expression during mouse gut development are summarized in figure 1. LPH gene expression in wild-type mice is highest before birth and declines during the weaning transition. In the absence of Hnf1α we show that *LPH* is reduced

(~50%) during development, however declines to undetectable levels after weaning. In contrast, inactivation of Gata4, does not effect the *LPH* mRNA levels before weaning. These data show a differential requirement for Gata4 and Hnf1α in the expression of LPH during development. To determine if this differential is gene-specific we also measured *Fabp1* gene expression levels in the two knockout models. The wild-type expression patterns of Fabp1 and LPH are very similar, which may suggest similar regulatory mechanisms. In this chapter, however we show that in contrast to the effects on LPH expression, Fabp1 is reduced before weaning in both knockout models. Collectively these data show that Gata4 or Hnf1α are partially required for *LPH* and *Fabp1* gene expression before weaning, which differs with their indispensability for *LPH* and *Fabp1* gene expression after weaning as concluded in chapter 3 and 4. To define the role of Hnf1α and Gata4 in the initiation of *SI* gene expression during weaning we also determined SI levels in the two knockout models. We found no distinguishable difference between knockout and wild-type expression, showing that Gata4 and Hnf1α are dispensable for SI gene expression during this phase of development.

In addition, this chapter defines the mRNA and protein expression of Gata4 and Hnf1α during intestinal development in great detail. We show that *Gata4* and *Hnf1α* mRNA levels increase in the small intestine during cytodifferentiation and show by immunofluoresence co-expression in the nuclei of E16.5 epithelial cells. After birth, we show a unexpected discordant relationship between mRNA levels and nuclear protein during a narrow developmental window, between P7 and P14. In this time high mRNA levels contrast with undetectable low protein levels for both Gata4 and Hnf1α. Together in Chapter 6 a collection of data is presented showing 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, these transcription factors are only partially required for the same genes prior to the final maturation that occurs at weaning, implicating other mechanisms in the development of gut function.

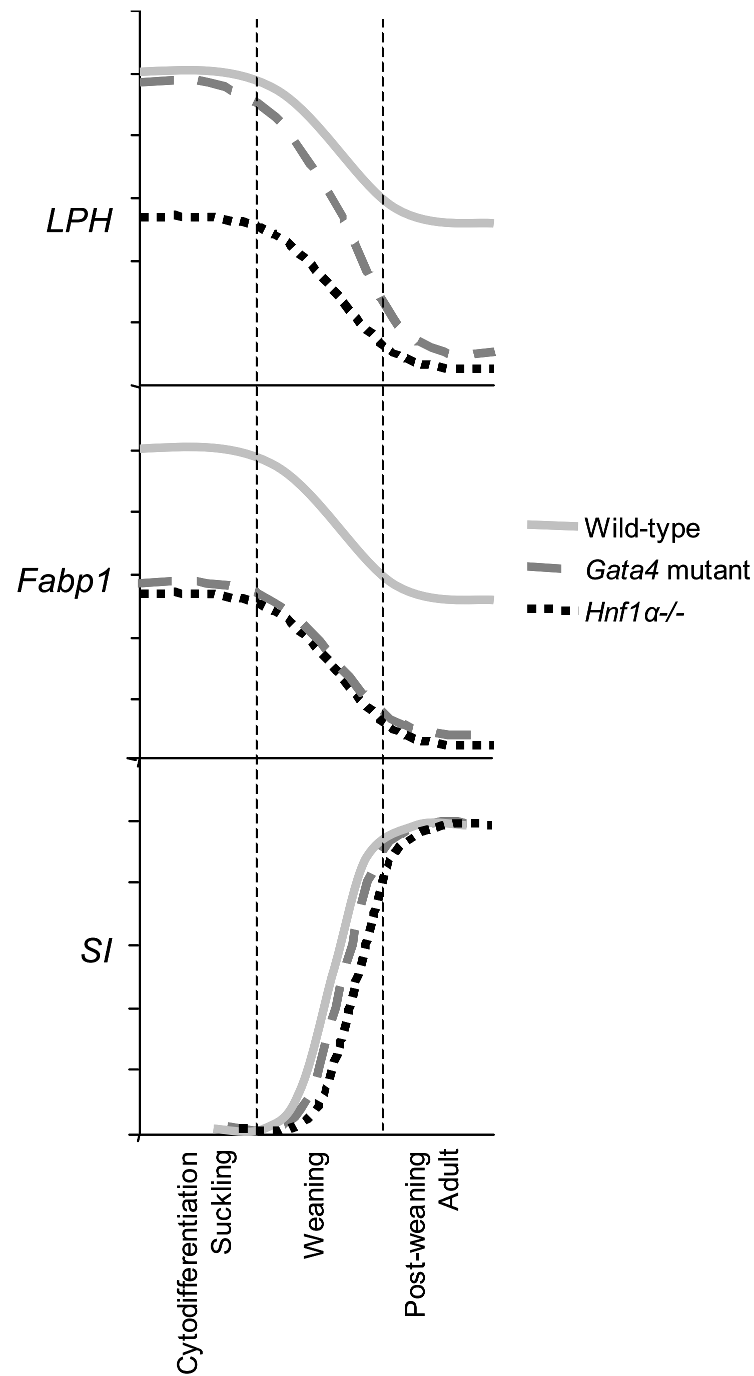


Fig. 1. Summary of Gata4 and Hnf1α regulation of LPH, Fabp1 and SI gene expression during intestinal development. Schematic representation of *LPH* (*top*), *Fabp1* (*middle*), and *SI* (*bottom*) gene expression in wild-type, Gata4 mutant, and Hnf1α-/- during development.

7.1.5 Intestinal Gata4 repression is mediated through FOG interactions

In **chapter 6** we defined the role of Gata4 and FOG co-factors in the determination of the proximal boundary of the ileal gene, apical sodium dependent bile acid transporter (*Asbt*), in the mouse small intestine. We demonstrate that after inactivating Gata4 before weaning, the horizontal expressing of Asbt extends until the proximal jejunum, indicating a critical role for Gata4 in the establishment of the proximal boundary during weaning. We further show that the Gata4 dependent *Asbt* repression can be recapitulated in CT-26, a cell line isolated from a mouse adenocarcinoma of the colon with ileal-like absorptive enterocyte features. In these cells, Gata4 over-expression resulted in the repressed activity of a 1.1kb *Asbt* promoter-reporter construct that contains 6 putative GATA binding sites, implicating that the Gata4 repression acts through this first 1.1kb promoter sequence.

FOG co-factors have been shown to physically interact with the N-terminal zinc finger of GATA family member, mediating GATA functions in several organ systems. In this chapter we aimed to identify those intestinal Gata4 dependent pathways that require the interaction with FOG co-factors. We first analyzed the expression of FOG co-factors (*Fog1* and *Fog2*) in the intestine and found that the intestinal expression of *Fog1* is highest and follows Gata4 expression, providing a topographic basis for functional interactions. As an experimental model we made use of a *Gata4* knock-in mouse line that has a single amino-acid substitution disrupting the binding with FOG co-factors(5). In this chapter we show that we were capable of integrating our conditional, inducible *Gata4* mutant with the mutant *Gata4* knock-in mouse line to establish an intestinal, inducible *Gata4* knock-in mouse. Utilizing this mouse model we determined the role of FOG co-factors in the intestinal Gata4 functions by comparing the phenotype of the intestinal knock-in mice with the intestinal *Gata4* mutant as described in chapter 4. In the *Gata4* knock-in animals we did not recognize a change in goblet cell number, indicating that the role of Gata4 in cell fate allocation is FOG independent. These mice also did not show changes in *LPH* expression, indicating that the activating role of Gata4 on this gene is also independent of FOG co-factors. The expression of *Asbt* however was induced in the jejunum of the knock-in mice, demonstrating a role for the interaction with FOG co-factors in this intestinal Gata4 function. Together these data indicate that the induction of ileal genes in the jejunum of adult mice by Gata4 is mediated, in part, through interactions with FOG co-factors.

7.1.6 A model of intestinal gene regulation by Gata4 and Hnf1α in vivo

The use of mouse knock-out models for both Gata4 and Hnf1α in this thesis has extended our understanding of the role that these factors play in intestinal specific gene regulation. A model has emerged from these studies in which Gata4 plays a central role in directing position-specific gene expression through specific interactions with other regulatory proteins, such as Hnf1α and FOG co-factors. In this model intestinal Gata4 functions are subdivided in 3 pathways (Fig. 2).

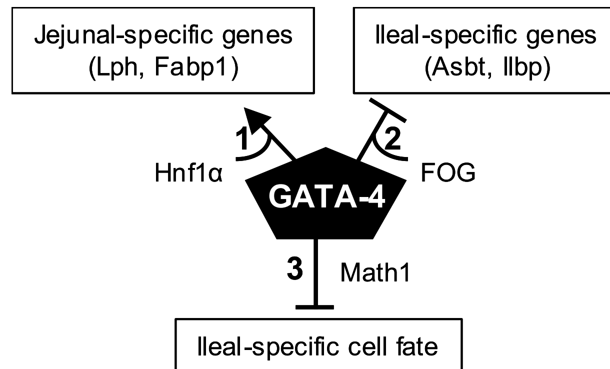


Fig. 2. Model depicting the 3 major Gata4 dependent pathways that are active in the adult jejunum to maintain jejunal-ileal identities. We hypothesize that (**1**) Gata4 and Hnf1a cooperatively activate jejunal-specific genes, (**2**) Gata4 interacts with FOG co-factors to repress ileal-specific genes and (**3**) inhibits ileal-specific cell fate through *Math1* regulation.

In the first pathway (*1*), consistent with previous cell-culture data, Gata4 and Hnf1α are both required for the activation of jejunal-specific genes in the adult mouse (**chapters 3&4**). We hypothesized based on cell-culture experiments described in **chapter 2** that the physical interaction between Gata4 and Hnf1α with each other and the proximal promoter results in synergistic activation of these genes. In the second pathway (*2*), Gata4 plays a critical role in the repression of ileal genes in the absorptive enterocytes of the adult mouse jejunum (**chapter 4**). We hypothesized based on experiments described in **chapter 6** that this Gata4 dependent repression is mediated, in part, through interactions with FOG co-factors and likely also requires interactions with the proximal promoters of these genes. The third pathway (*3*) represents the role of Gata4 in determining goblet cell number and enteroendocrine cell distribution between jejunum and ileum. Based on the increase in *Math1* expression in the Gata4 mutant mice, we hypothesize that Gata4 inhibits ileal-specific cell-fate by mediating Math1 levels, the underlying mechanism for this pathway however remains to be elucidated. An important consideration in this model is addressed in **chapter 5** in which we show that the requirement for Gata4 and Hnf1α is limited to the regulation of gene expression in the adult intestine. We therefore hypothesize that other regulatory proteins are essential for the expression of intestinal genes before weaning. In the future perspective section we describe studies that are directed to a better understanding of each of these pathways, and experiments are proposed that will define new factors involved in the complex regulation of intestinal gene expression.

7.2 Discussion of future perspectives

7.2.1 To define the importance of Gata4/Hnf1α interactions in vivo

From a mechanistic point of view it will be interesting to further define the differential regulation observed *in vitro* between Gata4 and Gata5 (**chapter 2**), however since Gata4 in the absorptive enterocytes is co-expressed with Hnf1α it is more relevant to further define the mechanism of Gata4/Hnf1α cooperativity. To ultimately characterize the function of the interaction between Gata4 and Hnf1α a knock-in mutation in either Gata4 or Hnf1α that disrupts this interaction will be necessary. The fact that the same domains required for the protein-protein interaction (zinc fingers of Gata4 /homeo- and pou-domain of Hnf1α) are also required for DNA binding is complicating this approach. In order to distinguish these functions, the future challenge will be to define amino-acids in either Gata4 or Hnf1α that are responsible for protein-protein interaction but do not play a role in DNA binding. To avoid redundancy among the different intestinal GATA factors, knocking in a mutation in the Hnf1α protein that disrupts binding to all intestinal GATA factors would be particularly appealing. We currently know that the homeo- and pou-domains of Hnf1α are necessary for the interaction with GATA factors; however, these domains together are ~200 amino-acids long and a random point mutation approach would therefore be costly and cumbersome. To minimize this region a series of deletion-constructs need to be tested in protein-protein interaction assays (such as GST-pulldown assays). When the minimal domain for the interaction is reasonably small (<50 amino-acids) an altered-specificity mutant approach as described previously (FOG:GATA interaction (4)) can be employed to determine the key amino-acids for the GATA/Hnf1α interaction (Fig 3.). In this experiment Hnf1α mutants will be isolated that are specifically impaired for interacting with Gata4 using a split yeast-two-hybrid system. Out of frame mutations, multiple mutations and mutations that also disrupt DNA binding are excluded from further analysis. The remaining point-mutation will subsequently be tested in cell-culture for their ability to activate the *LPH* promoter.

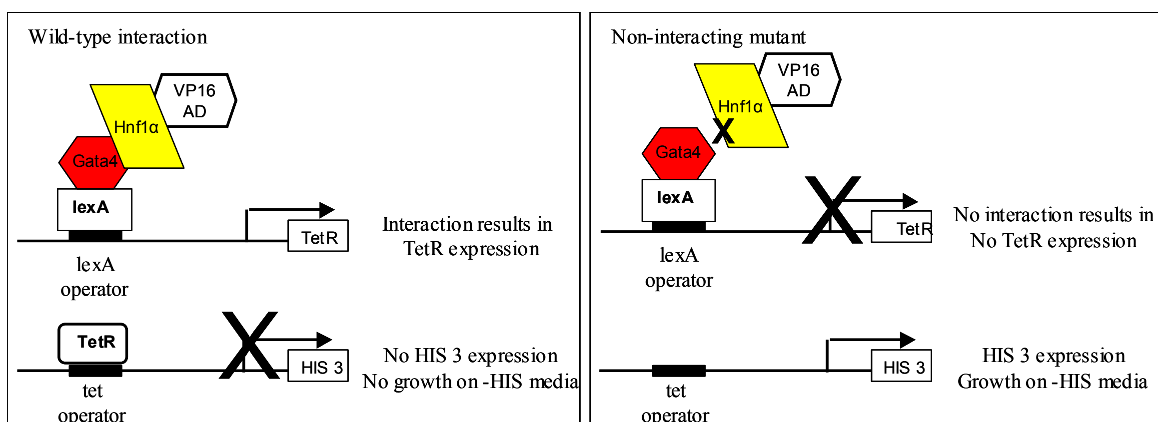


Fig. 3. Schematic depiction of the split two-hybrid screen. When an Hnf1α mutant cannot interact with Gata4, TetR will not be repressed, resulting in growth on His negative media. Growth therefore indicates the presence of Hnf1α mutations that disrupt the interaction with Gata4, and will be further analyzed. Tet R, Tet repressor; VP16 AD, VP16 activation domain; lexA, lexA DNA binding domain

An alternative approach to determine critical amino-acids required for the Hnf1α/Gata4 interaction is to crystallize the Hnf1α/Gata4/DNA complex. The crystal-structure will reveal contact point between Hnf1α with Gata4 and the DNA. With this approach it is possible to predict critical amino-acids responsible for the protein-protein interactions. It will be essential to add DNA to the crystal-structure, since this will provide clues which amino-acids are more likely responsible for protein-protein interaction and which are also involved in DNA binding. The current research group has already established a collaboration with a crystallographer (dr. Young-In Chi, University of Kentucky) who has already crystallized the Hnf1α/DNA complex (3).

7.2.2 To define the role of HNF-1β in SI gene expression

This thesis clearly demonstrates that Gata4 and Hnf1α are dispensable for the expression of endogenous *SI* message levels in the mouse small intestine (**chapter 3,4 and 5**). This was an unexpected finding, since it is in contrast with many previous studies using cell culture and other *in vitro* experiments that suggest Gata4 and Hnf1α are activators of the *SI* gene. These earlier studies especially point to an important regulatory role for Hnf1α in the gene expression of *SI*. Convincing evidence came from a study in which transgenic mice harboring a *SI* promoter with a mutated HNF-1 site (SIF3) showed a reduction in transgene expression relative to transgenic mice with a wild-type *SI* promoter (2). In light of our results showing that Hnf1α is dispensable for *SI* gene expression (chapter 3), we hypothesize that this SIF3 mutation interrupts the binding of another critical activator, possibly Hnf1β. Although the ability of Hnf1β to activate the *SI* promoter in cell culture models is minimal, it binds to SIF3 *in vitro* and is thus a candidate activator of *SI* gene expression *in vivo*. To test the hypothesis that Hnf1β can activate the *SI* gene in the absence of Hnf1α, it will be necessary to generate mice that lack the expression of both Hnf1α and Hnf1β in the intestine. Since germline *Hnf1β* null mice die very early during embryogenesis and *Hnf1α* null mice are not fertile, a conditional approach will be necessary. Using *CreloxP* recombination technology, it is possible to generate mice with floxed *Hnf1α* and *Hnf1β* alleles and to cross these with the established inducible villinCreER^{T2} line as described in this thesis (**chapters 4&5**). Such a mutant mouse line will help decipher the redundancy in function between Hnf1α and Hnf1β and will contribute to a better understanding of the complex regulation of *SI* gene expression in the small intestine.

7.2.3 To define the functional domains of Gata4 in vivo

In this thesis we made use of *CreloxP* technology to generate an intestinal, inducible *Gata4* mutant mouse line (**chapter 4**). To generate this line a floxed *Gata4* line was used in which *exon2,* containing the translational start site and the C-terminal activation domains, were flanked by *loxP* sites. Crossing this line with an inducible villinCreER^{T2} line, resulted in intestinal specific recombination of the floxed exon. Interestingly, due to the floxing strategy, a truncated Gata4 species was still being transcribed and translated as shown by western blot (**chapter 4**), likely due to an in frame ATG in *exon 3*. Since the zinc fingers and basic region of this truncated *Gata4* species are still intact, the capacity to bind DNA was not affected. The mutant Gata4 mouse line can therefore better be defined as an intestinal, inducible activation domain deletion (act. dom. del.) of Gata4. Consequently the phenotype of this mouse maps the function of the activation domains of Gata4 and does not reveal Gata4 functions that lie outside of the activation domains. Therefore, future studies are on the way to generate a *"complete"* intestinal *Gata4* knock-out by the use of a floxed *Gata4* line that does not result in the translation of a Gata4 mutant. Important conclusions regarding Gata4 function can be drawn from the comparison between the *Gata4* null mice with the *Gata4* act. dom. del. mutant that is described in this thesis. There are 3 possible outcomes that can be anticipated from this comparison: 1) the phenotype of the *Gata4* null mice is identical to the *Gata4* act. dom. del. mutant, 2) the phenotype is stronger and 3) there is no phenotype. If the first scenario is true (phenotype is identical), we can conclude that all the functions of Gata4 in the intestine are dependent on the presence of intact activation domains. In the second situation (phenotype is stronger), the functions of activation domains and the rest of the molecule can be dissected. For example new phenotypic changes that are present in the *Gata4* null mouse, but not in the *Gata4* mutant, can be mapped to functions outside the activation domains of Gata4. Finally it is possible that the phenotype of the intestinal *Gata4* null mouse is indistinguishable from wild-type, in this scenario compensatory effect (for example by Gata6), that could not occur in the mutant, would have to be considered. During this thesis a valuable collaboration with dr. William. T. Pu (Children's Hospital Boston, department of cardiology) has been established; that laboratory is currently generating a new *Gata4* floxed mouse line designed to knockout *Gata4* completely. These mice will be used to cross with the inducible villinCreER^{T2} line, resulting in an intestinal, inducible *Gata4* null mouse line. Taken together the generation of an intestinal *Gata4* null mouse would add significantly to our understanding of the function of Gata4 in the adult small intestine.

7.2.4 Reverse the curse – To delineate the plasticity of the ileum by over-expressing Gata4 in vivo

Chapter 4 elegantly shows the plasticity of the adult jejunal epithelium to change into an ileal like epithelium in the absence of active Gata4. This finding not only shows that Gata4 is required for the maintenance of the normal jejunum epithelium, but in addition reveals that the presence

of Gata4 inhibits an ileal phenotype. This raises the question whether the reverse is also true; can Gata4 also independently activate a jejunal program in the ileum? We hypothesize that over-expression of Gata4 in the ileum will result in a shift towards a jejunal phenotype. To test this hypothesis an over-expression model will be necessary in which Gata4 can be over-expressed in the adult ileum upon induction. A possible approach to accomplish such a model is depicted in fig. 4.

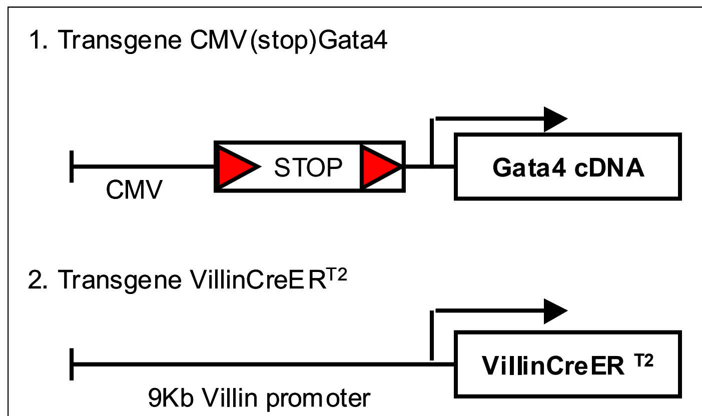


Fig. 4. Schematic representation of the transgenic mice required to generate an intestinal inducible, Gata4 over-expression mouse line. (1) Transgene containing a constitutive active promoter (such as CMV) with a floxed STOP cassette fused to the mouse cDNA has to be established. (2) VillinCreER^{T2} transgene. The offspring that is positive for both transgenes will over-express Gata4 in the intestine after induction with Tamoxifen^{TM}.

First a transgenic mouse expressing Gata4 under the control of a constitutive active promoter with an inserted floxed stop cassette has to be generated. Next this transgene can be crossed with the inducible villinCreER^{T2} line resulting in an intestinal, inducible Gata4 over-expression model. This model will test the plasticity of the ileal epithelium to convert to a jejunal phenotype and will further confirm the ability of Gata4 to act as a positional factor. However when the outcome of this model is that the ileum does not change into a jejunal phenotype, it can be concluded that the mechanism responsible for maintenance of a jejunal phenotype by Gata4 requires other factors that are absent in the ileum. Or this can be explained by the presence of ileal specific factors that can repress the activation of jejunal genes by Gata4. A pitfall of this mouse model is that Gata4 will also be over-expressed in the colon, this consequence however may also become interesting in revealing a potential role of Gata4 in maintaining a small intestinal phenotype. Although the generation of such a Gata4 over-expressing transgene is a considerable investment, this approach will provide key *in vivo* data towards a better understanding on the mechanism by which Gata4 maintains the jejunal-ileal differences.

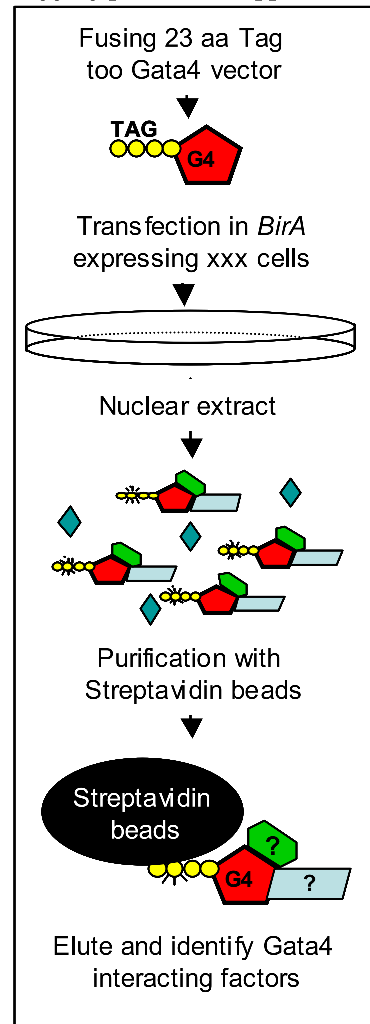
7.2.5 To define the potential physiological benefits of inactivating Gata4 in an ileal resection model

Loss of ileum function can occur when a patient suffers from inflammatory bowel disease (IBD), radiation enteritis, HIV, cystic fibrosis or is due to ileal resection. This loss of ileal function, results in a defect in ileal bile acid transport which can not be compensated by the rest of the small intestine and consequently results in considerable morbidity. Bile acid malabsorption in these circumstances leads to bile-salt induced diarrhea and gallstone formation. More importantly, bile acid malabsorption may be a risk factor for the development of colorectal cancer (8). Various animal studies, including a rat ileal resection model, support this proposition (7). Furthermore, apical sodium-dependent bile acid (Asbt) transport activity and Asbt protein expression are decreased in rabbit models of ileal inflammation(10). In **chapters 4** and **6** of this thesis we show that genes responsible for the bile-acid transport can be activated in the jejunum when Gata4 is inactivated. We therefore hypothesize that inactivation of Gata4 in the jejunum may rescue ileal function after ileal resection or ileal inflammation. To test this hypothesis we propose to perform ileal-resection surgery on our mutant *Gata4* mice in which we subsequently inactivate Gata4. A control group will be necessary to include in which we do not inactivate Gata4, but do perform the surgery. When the experimental mice are recovered from their surgery they will be analyzed for indicators of bile acid metabolism and fat metabolism such as stool fat and bile acids, bile acid pool size, and liver cholesterol hydroxylase. Analogous to these experiments an IBD mouse model can also be established in which Gata4 can be inactivated upon tamoxifen treatment. The same indicators will be used to test the status of the bile acid and fat metabolism in control and *Gata4* mutant IBD mice. Together these experiments will demonstrate if the inactivation of Gata4 and the subsequent induction of ileal genes in the jejunum can rescue ileal function. This experiment will carry the findings as described in this thesis to the next level, and will test the ability of function recovery through the disruption of Gata4 dependent repression. During the preparation of this thesis we have already started optimizing ileal surgery on adult mice in collaboration with dr. T. Buckmiller (department of surgery, Children’s Hospital Boston) and dr. B.L. Shneider (division of pediatric hepatology, Mount Sinai Medical Center, New York).

7.2.6 To identify new factors that interact with Gata4 using a biotinylation tagging proteomic approach

In **chapter 4** we show that Gata4 can be both an activator and a repressor of different target genes in the same cells. In this chapter we discuss the parallels with Gata1 regulation in heamotopoetic cells, in which it is well established that Gata1 can be both a positive and negative regulator in the same cells dependent on the context of the promoter and the available co-factors. In **chapter 6** we show that the Gata4 dependent repression is mediated, in part, through interactions with FOG co-factors a novel intestinal knock-in mouse model. We therefore hypothesize that Gata4 forms an inhibitory complex on the promoter of *Asbt* to repress this gene in the jejunum. To identify the factors that are part of this inhibitory complex we propose a biotinylation tagging/proteomics approach in CT-26 cells similar to the approach used earlier for Gata1 (9). This procedure makes use of biotin/avidin binding which is the strongest noncovalent interaction known in nature. As schematically depicted in figure 5, it will require the tagging of a mouse Gata4 construct with a small (23 amino acid) peptide sequence to its N-terminus that can be biotinylated by BirA biotin ligase. When this construct is co-transfected in CT-26 cells that stably express BirA, the Gata4 tag proteins will be effectively biotinlyated. This allows a single-step purification of biotinylated Gata4 and its interacting proteins using streptavidin beads. The Gata4 complex can subsequently be analyzed for novel interacting partners of Gata4 by western analyses. In this approach the known and well described Gata4/Hnf1α interaction can serve as a positive control, as well as the Gata4/FOG interaction. Functional data of novel factors can next be obtained by co-transfection with Gata4 and eventually turn back to *in vivo* approaches using knock-out mouse approaches. This experimental procedure will therefore provide fundamental knowledge on the mechanisms by which Gata4 performs overlapping functions to maintain the jejunal-ileal differences in the adult small intestine.

Fig. 5. **Schematic of biotinylation tagging/proteomics approach**



7.2.7 To identify factors required for LPH gene expression before weaning using DNase hypersensitivity assays in vivo

One of the major conclusions that can be drawn from this thesis is that *LPH* gene expression is differentially regulated before and after weaning. The transcription factors Gata4 and Hnf1α are both required for *LPH* gene expression after weaning however before weaning and during cytodifferentation Gata4 is dispensable and Hnf1α is only partially required (**chapter 5**). This presents us with a paradox in which the two best described and critical activators of the *LPH* gene in the adult intestine, are both dispensable during the first weeks of life when lactose digestion is critical for survival. We therefore hypothesize that redundant mechanisms are present before weaning, "protecting" the *LPH* gene for not being transcripted during this critical time. Using the same rationale, we could hypothesize that redundant mechanisms "protect" the expression of *SI* gene expression in the adult small intestine. This hypothesis implies that factors other than Gata4 and Hnf1α are capable of activating *LPH* before weaning, but are not acting as critical activators after weaning. Cells in culture will not be adequate to identify these factors, because it requires an experimental model in which weaning naturally occurs. We therefore propose to conduct DNase hypersensitivity assays using mouse epithelial extracts, to identify *cis*-acting elements in the *LPH* regulatory region that bind proteins before weaning but not after weaning. In this approach DNase hypersensitive sites (DHS) on the 5'-flanking region of *LPH* will be compared from 14 day old and adult wild-type mice. The known binding sites of Gata4 and Hnf1α on the proximal promoter, will serve as a positive internal control and will give a strong DHS in the adult extracts. Using this experiment DHS that are present before weaning, but not after weaning will be further investigated using other DNA binding assays, such as DNA footprinting and EMSA's. This will lead to the identification of those *cis*-acting elements that are binding proteins before weaning and not after weaning. The use of DNA sequence analysis software, such as Mapper™ , will subsequently help identify those proteins that display differential binding during weaning. The potential to activate the *LPH* promoter by these novel proteins can be analyzed in the established cell-culture systems and eventually be tested in mouse knock-out systems. This experiment will provide crucial information on the mechanism that orchestrate the expression of *LPH* before weaning, and consequently the molecular mechanism underlying weaning.

7.2.8 To define the molecular pathways of intestinal differentiation regulated by Gata4, Hnf1α, and FOG cofactors in vivo using gene expression profiling

The precisely coordinated spatial and temporal control of intestinal gene expression necessary for development and differentiation is likely the result of the interplay of multiple regulatory mechanisms. The analyses of the phenotypes of our knock-out models in this thesis has been biased towards hypothesized target genes based on previous cell-culture data. Some findings, such as the shift in cell-fate in the *Gata4* mutant mice, were subtle and could easily have been

missed. A more effective and unbiased method to define regulatory pathways is to conduct high-quality gene profiling on our validated mouse models. Recently, the Children's Hospital Informatics Program (CHIP) has integrated a genome-wide binding analysis (6) with mRNA expression data and existing functional annotations to quantify the likelihood that genes with varying degrees of similarity in mRNA expression profiles and function will be regulated by a common mechanism (1), and found that the combination of expression data along with functional annotations provides a better predictive model of co-regulation than either data source alone. Using this combined model together with our phenotypic intestinal knock-out models for Hnf1α, Gata4$^{\Delta ex2/\Delta ex2}$ and the Gata4$^{\Delta ex2/ki}$ mutant model, we will be able to define common and divergent pathways of Gata4 and Hnf1α gene regulation, the involvement of Gata4 in complex cell fate pathways, and the subset of pathways that require FOG co-factors. These studies will provide important data on some of the molecular networks critical for intestinal function. The hypothesis to be tested in this experiment is that Gata4 regulates the differences between jejunum and ileum through multiple signaling pathways involving interactions with Hnf1α and FOG co-factors. The combination of our knockout/knock-in models with gene expression profiling provides a unique opportunity to define in detail complex, Gata4 dependent biochemical pathways necessary for intestinal function. Individual gene analysis will define specific intestinal target genes that are affected by the absence of Gata4 or Hnf1α, whereas cluster analysis will indicate signaling pathways affected by the absence of Gata4 or Hnf1α. An alteration in the same subset of genes by the absence of Gata4 and Hnf1α would support a mechanism by which these two transcription factors interact, whereas an alteration in a divergent subset of genes would suggest that Gata4 and Hnf1α do not cooperate. Likewise, integration of the intestinal *Gata4* knock-in mice will define the subset of Gata4 pathways regulated by FOG co-factors. The strengths of these experiments is the use of the novel knockout/knock-in models and the specific expertise of the Children's Hospital Informatics Program with transcription factor activation of target genes, and thus, there is a high likelihood that these experiments will produce valuable data on critical pathways in the small intestine.

7.3 Significance

The studies presented in this thesis, and the concepts which emerged, describe unique aspects of intestinal specific gene regulation by transcription factors Gata4 and Hnf1α. They also set the stage for future studies designed to delineate details of the regulation of intestinal gene expression and differentiation by Gata4, Hnf1α and other factors as described in the future perspectives. This thesis represents the most recent work of the continuing research that started in the late nineteen eighties by cloning the lactase-phlorizin hydrolase gene and has now evolved into understanding the complex nature of molecular pathways that orchestrate maintenance of intestinal differentiation in the postnatal gut. The fundamental understanding in these molecular

pathways will serve as a basis for identifying new avenues in therapeutic interventions that will significantly improve the management of patients with intestinal diseases.

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CHAPTER 8

Samenvatting

SAMENVATTING

In dit proefschrift is de rol die de transcriptiefactoren Gata4 en Hnf1α spelen in de regulatie van darmspecifieke genexpressie in muizen bestudeerd, gebruikmakend van markers voor differentiatie en ontwikkeling van de dunne darm; *LPH* en *SI*. **Hoofdstuk 1** geeft een overzicht van de huidige kennis op het gebied van darmdifferentiatie en -ontwikkeling en beschrijft in detail het onderzoek naar de complexe mechanismen, die betrokken zijn bij darmspecifieke genexpressie, dat voorafgaand in zowel het laboratorium van R.J. Grand en S.D. Krasinski, als andere laboratoria is uitgevoerd. In dit hoofdstuk worden experimenten beschreven, die laten zien dat LPH en SI perfecte markers zijn voor het differentiatieproces van de dunne darm. LPH en SI zijn suikereiwitten, die verankerd zijn in het membraanoppervlak van enterocyten (absorptieve cellen) en verantwoordelijk zijn voor de vertering van respectievelijk lactose (het belangrijkste koolhydraat in melk) en α-disachariden (voornaamste suikers in vaste voeding). De ontwikkeling van de darm kan verdeeld worden in verschillende belangrijke fases, die samenvallen met veranderingen in *LPH* en *SI* genexpressie. Eerder werk heeft laten zien, dat de eiwitexpressiepatronen van LPH en SI parallel lopen aan die van hun messenger RNA, pre-mRNA en hun transcriptionele activatie zowel wat betreft de horizontale gradiënt als de ontwikkelingsgradiënt. Verder hebben studies met transgene muizen laten zien, dat de informatie voor adequate verticale, horizontale en ontwikkelingsexpressiepatronen van zowel *LPH* als *SI* gecodeerd is in de 5′-flanking regio van de promoters van deze genen. Al deze onderzoeksresultaten wijzen uit, dat de synthese van LPH en SI hoofdzakelijk gereguleerd wordt op transcriptieniveau. Recentelijk zijn er in de DNA sequenties van de proximale promoters van *LPH* en *SI* geconserveerde bindingselementen gevonden, die zowel dicht naast elkaar als vlak bij de TATA-box gelegen zijn. Tussen deze sequenties zijn bindingsplaatsen gevonden voor de zinkvinger bevattende GATA familie en de homeodomein bevattende HNF-1 en Cdx families. Studies van ons laboratorium laten zien dat GATA en HNF-1 factoren met elkaar interacties kunnen aangaan, die nodig zijn om de *LPH* en *SI* promoters synergistisch te activeren *in vitro*. Deze fundamentele observatie door dr. Herbert M. van Wering, heeft geleid tot het centrale paradigma van dit proefschrift, dat de overlappende expressie van deze geconserveerde transcriptiefactor families gezamenlijk verantwoordelijk zijn voor de darmspecifieke expressie van bepaalde target genen *in vivo* en daarmee ook wellicht van belang zijn voor darmdifferentiatie en de groei van de darm. In dit proefschrift wordt met behulp van knock-out muismodellen de rol van Gata4 en Hnf1α voor darmspecifieke genexpressie bestudeerd. De resultaten van deze studies zijn een betekenisvolle toevoeging aan het huidige begrip van de moleculaire mechanismen die darmdifferentiatie, -ontwikkeling en -functie reguleren.

8.1 Gata4 is de belangrijkste GATA factor voor LPH genexpressie.

Experimenten in **hoofdstuk 2** laten zien, dat Gata4 een belangrijke GATA factor is in de dunne darm, die bindt aan het GATA element van de muis *LPH* promoter en dat het expressiepatroon

van *LPH* in het jejunum en ileum correleert met dat van Gata4. Om een rol voor Gata4 in de regulatie van *LPH* expressie aannemelijker te maken, is de relatie tussen het expressiepatroon van het *LPH* mRNA en de expressie van Gata4 eiwitten langs de lengte van de dunne darm bepaald door middel van kwantitatieve RNAse protection asssays (RPA), western blots en kwalitatieve immunohistochemie (IHC). Het expressiepatroon van het Hnf1α eiwit is ook bepaald, aangezien eerdere studies hebben laten zien, dat Gata4 in combinatie met Hnf1α darm genen kan activeren in celkweken. Hnf1α eiwit is aanwezig in de kernen van de enterocyten van het villusepitheel en is in gelijke mate langs de gehele lengte van de dunne darm verdeeld. Gata4 eiwit is aangetoond in de kernen van de enterocyten van de villus maar ook in de ongedifferentieerde enterocyten in de crypten en is nauwelijks detecteerbaar in het distale ileum. Het aflopende patroon van Gata4 eiwit naar het ileum toe correleert met het lage niveau van *LPH* mRNA in deze regio. Collectief suggereren deze experimenten, dat Gata4 de belangrijkste GATA factor is voor *LPH* genexpressie in de volwassen dunne darm.

Daarnaast staat er in **hoofdstuk2** beschreven hoe Gata4 zowel zonder Hnf1α als met Hnf1α de menselijke *LPH* promoter kan activeren. Deze mechanisme zijn *in vitro* onderzocht gebruikmakend van HeLa cellen. Er is gekozen voor HeLa cellen, aangezien deze zelf geen Hnf1α en GATA factoren produceren, en daarmee uitermate geschrikt zijn voor gebruik in experimenten om een beter begrip van de regulatiemechanismen te verkrijgen. Er is gevonden dat Gata4 de *LPH* promoter samen met Hnf1α synergistisch activeert, vergelijkbaar met de interactie tussen Gata5 en Hnf1α. Deze activatie gaat via een mechanisme, waarbij GATA-bindingsplaatsen en de Gata4 activatiedomeinen niet noodzakelijk zijn. De fysieke interactie tussen Gata4 en Hnf1α evenals een intacte HNF-1-bindingsplaats zijn wel noodzakelijk voor de Gata4/Hnf1α synergie. De C-terminale zinkvinger van Gata4 medieert de fysieke interactie met Hnf1α. Daarnaast is gevonden dat Gata4, in tegenstelling tot Gata5, ook in staat is de *LPH* promoter te activeren zonder de aanwezigheid van Hnf1α. Deze Hnf1α onafhankelijke activatie verloopt via een mechanisme waarvoor intacte GATA-bindingsplaatsen en GATA-activatiedomeinen noodzakelijk zijn. In een volgend experiment zijn op elegante wijze domeinen gewisseld tussen Gata4 en Gata5, waaruit blijkt dat de zinkvinger-domeinen en de basale regio van Gata4 verantwoordelijk zijn voor de Hnf1α-onafhankelijke activatie. Gel-shift experimenten tonen vervolgens aan, dat de verschillen tussen Gata4 en Gata5 niet te verklaren zijn door affiniteitsverschillen met de GATA-bindingsplaatsen. Uit deze data kan worden geconcludeerd, dat Gata4 de voornaamste GATA factor is voor de regulatie van *LPH* genexpressie en dat Gata4 niet alleen in staat is om de *LPH* promoter te activeren in samenwerking met Hnf1α, maar ook in staat is om de *LPH* promoter te activeren zonder Hnf1α.

8.2 Hnf1α reguleert een kleine groep genen in het jejunum van volwassen muizen.

In **hoofdstuk 3** hebben we de celkweekmodellen, die afhankelijk zijn van over-expressie van de transcriptiefactoren in een extra-chromosomale context achter ons gelaten, om ons vervolgens te richten op de karakterisatie van het mechanisme waarmee Hnf1α darmspecifieke genen reguleert

met behulp van *Hnf1α* knock-out muizen. Genexpressieniveaus van zowel *LPH* als *SI* en andere mogelijke darmspecifieke Hnf1α target genen zijn bepaald in het jejunum van volwassen *Hnf1α* nul muizen en vergeleken met de niveaus in wild-type muizen. De hoeveelheid mRNA van *LPH* en *SI* is gekwantificeerd door middel van RPAs en real-time RT-PCR. Hieruit is gebleken dat *LPH* mRNA in de *Hnf1α* knock-out muizen 95% lager is dan de *LPH* mRNA niveaus gemeten in wild-type controle muizen ($P<0.01$, $n=4$). Dit is het eerste bewijs dat Hnf1α onmisbaar is voor de expressie van *LPH* in de volwassen muis dunne darm *in vivo*. Verassend is, dat de *SI* mRNA niveaus langs de gehele dunne darm niet lager zijn in de afwezigheid van Hnf1α in zowel de pasgeboren als in de volwassen dunne darm van de knock-out muizen. Dit is een onverwacht resultaat gezien de grote hoeveelheid literatuur, die wijst op een rol voor Hnf1α in de activatie van *SI* genexpressie. Uit deze resultaten kan worden geconcludeerd, dat Hnf1α onmisbaar is voor *LPH* expressie *in vivo*, maar dat Hnf1α niet noodzakelijk is voor *SI* genexpressie in de volwassen dunne darm van muizen.

Verder wordt er in **hoofdstuk 3** beschreven hoe wij chromatin immunoprecipitatie assays (ChIP) hebben toegepast op epitheelcellen uit muisdarmen. De ontwikkeling van deze techniek heeft het mogelijk gemaakt om eiwit-DNA interacties en modificaties aan histonen te bestuderen in een *in vivo* context. Wij zijn de eerste, die deze techniek hebben gebruikt op epitheelcellen uit muisdarmen en laten daarmee voor het eerst zien, dat Hnf1α bindt aan de proximale promoters van *LPH* en *SI* in de volwassen dunne darm *in vivo*. Het is opmerkelijk dat ondanks de verbinding die Hnf1α aangaat met de *SI* promoter, Hnf1α niet verantwoordelijk is voor de activatie van de transcriptie van *SI*. Terwijl de verbinding van Hnf1α met de proximale promoter van *LPH* wel correspondeert met de onmisbaarheid van Hnf1α voor de activatie van dit gen. Deze discrepantie is eerder gevonden in genen van de lever en pancreas (1). Vervolgens is door middel van ChIP-assays aangetoond dat de acetylatietoestand van de histonen die geassocieerd zijn met de proximale promoters van *LPH* en *SI* onveranderd blijven in de afwezigheid van Hnf1α. Dit wijst erop, dat Hnf1α niet betrokken is bij de acetylatie van deze histonen, dit in tegenstelling tot wat eerder gevonden is voor genen in de lever en pancreas. Ten slotte is in dit hoofdstuk door middel van semi-kwantitatieve RT-PCR een groep potentiële Hnf1α target genen getest op hun expressieniveaus in het jejunum van volwassen en pasgeboren *Hnf1α* knock-out muizen. Er is gevonden dat een aantal genen afhankelijk is van Hnf1α voor hun expressie in het jejunum, zoals; *LPH*, *Fabp1*, *α_1-antitrypsine* and *guanylin*. Terwijl de expressie van andere genen zoals; *SI*, *Fabp2*, *aldolaseB*, *Hnf4α*, *SGLT1*, *L-PK* and *NeuroD1,* niet van Hnf1α afhankelijk is. Samengevat kan uit de data van dit hoofdstuk worden geconcludeerd, dat Hnf1α een activator is van een subgroep van darmspecifieke genen (inclusief *LPH*, maar niet *SI*) en dat deze Hnf1α-afhankelijke inductie van darmgenen niet door de acetylatie van histonen tot stand komt.

8.3 Gata4 is verantwoordelijk voor de instandhouding van de identiteitsverschillen tussen jejunum en ileum in de volwassen dunne darm.

In **hoofdstuk 4** is de functie van Gata4 in de volwassen muis dunne darm bepaald, gebruikmakend van een nieuw ontwikkeld conditionele knock-out muis model. Dit hoofdstuk start met het verhelderen van de bestaande discrepantie in de literatuur over de cellulaire expressie van Gata4 in het darmepitheel van muizen (met behulp van co-immunofluoresentie, co-IF). In overeenstemming met de literatuur is een sterk Gata4 signaal gevonden in de kernen van de enterocyten op de villus. Er is echter ook een niet eerder erkende Gata4 kleuring in het proliferatieve compartiment aangetoond door middel van dubbelkleuring met Ki67 (marker van proliferatieve cellen). Zwakke Gata4 expressie is ook aangetoond in de Paneth cellen, maar in de Goblet en enteroendocriene cellen is Gata4 duidelijk niet detecteerbaar. Verder is gevonden, dat Gata6 samen met Gata4 tot expressie komt in de enterocyten van het volwassen muis jejunum. Hieraan kan worden toegevoegd, dat Gata4 eiwitten afwezig zijn in het distale ileum van de mens. Deze geconserveerde afwezigheid van Gata4 in de volwassen distale dunne darm en het activatiepotentiaal van Gata4 liggen samen ten grondslag aan de hypothese, dat Gata4 als positionele factor functioneert bij het in stand houden van de verschillen tussen jejunum en ileum. Om deze hypothese te testen is het noodzakelijk om Gata4 te inactiveren in de volwassen dunne darm en daarom is een darmspecifieke induceerbare Gata4 knock-out muislijn ontwikkeld. In dit model is een eerder gevalideerde muislijn gebruikt, waarin een deel van exon2 van *Gata4* geflankeerd is met loxP sequenties (floxed). Deze *Gata4*$^{flox/flox}$ muislijn is gekruist met een andere eerder gevalideerde transgene muis (Villin*Cre*ER^{T2}), die de DNA-recombinase Cre tot expressie brengt in de epitheelcellen van de dunne en dikke darm. Deze Cre is gefuseerd aan een gemuteerde oestrogeen receptor en blijft hierdoor in het cytoplasma totdat het in aanraking komt met tamoxifen^{TM}, waardoor Cre naar de nucleus wordt getransporteerd en gefloxed DNA recombineert. Effectief resulteert een tamoxifen^{TM} behandeling in de inactivatie van Gata4 specifiek in de dunne darm van deze muizen.

Het fenotype van de mutant muislijn heeft nieuwe Gata4 functies onthult, die overeenstemmen met de hypothese dat Gata4 als positionele factor fungeert in de volwassen dunne darm. In het jejunumepitheel van de *Gata4* mutant zijn aanwijzingen gevonden voor ileum transformatie op basis van celcompositie en genexpressie. Eerder geïdentificeerde Gata4 target genen, die normaal hoog tot expressie komen in het jejunum, zoals *LPH* and *Fabp1*, komen in het Gata4 mutant jejunum laag tot expressie. Dit is kwalitatief aangetoond op eiwitniveau door middel van IF en kwantitatief op mRNA niveau met behulp van real-time RT-PCR (~10% en 20% van de controle, $P<0.05$). Daarentegen is er geen verschil gevonden in *SI* en *Fabp2* genexpressie in het jejunum van de mutant muizen, vergelijkbaar met de bevindingen in de *Hnf1α* knock-out muizen. Dit resultaat is eveneens in overeenstemming met de hypothese, aangezien *SI* en *Fabp2* beide ook tot expressie komen in het ileum. Een intrigerend resultaat is, dat er in de enterocyten van het Gata4 mutant jejunum een zeer aanzienlijke toename van de ileumspecifieke genen *Asbt* en *Ilbp* is gevonden. Dit is wederom bevestigd met behulp van IF en

real-time RT-PCR (20 maal verhoogd, P<0.01; en 644 maal verhoogd, P<0.001, respectievelijk). Het fenotype blijft niet beperkt tot de genexpressie van de enterocyten. Ook zijn er veranderingen gevonden in de Goblet en enteroendocriene populaties. In overeenstemming met een ileumtransformatie, is er een toegenomen aantal Goblet cellen per villus en een verschuiving in de compositie van de subpopulaties van enteroendocriene cellen in het jejunum van de *Gata4* mutant. Deze cellulaire verschuiving onthult een nieuwe functie voor Gata4, wat wellicht de aanwezigheid van Gata4 in het proliferatieve compartiment verklaard. Om dit fenotype nog beter te begrijpen zijn markers van de Wnt en Notch pathways geanalyseerd. Er is een significante toename in *Math1* genexpressie in het jejunum van de *Gata4* mutant (N=3, P<0.05). Aangezien Math1 verantwoordelijk is voor de formatie van de secretoire cellen in het darmepitheel, past deze bevinding goed bij het toegenomen aantal Goblet cellen. In conclusie worden er in dit hoofdstuk nieuwe onverwachte en fysiologisch belangrijke functies voor Gata4 in de dunne darm onthult, gebruikmakend van de nieuwste knock-out technologie. De resultaten van dit hoofdstuk zijn fundamenteel voor dit proefschrift en vormen de basis voor toekomstige studies die zullen worden uitgevoerd, zoals verder beschreven staat in **7.2 future perspectives**.

8.4 De rol van Gata4 en Hnf1α in de regulatie van genen tijdens differentiatie en ontwikkeling van de dunne darm.

In **hoofdstuk 5** wordt de rol van de transcriptiefactoren Hnf1α en Gata4 in de activatie en de instandhouding van *LPH, Fabp1* en *SI* genexpressie tijdens de differentiatie en groei van de dunne darm in ontwikkelende muizen onderzocht. De effecten op genexpressie in de ontwikkelende dunne darm na de inactivatie van Gata4 en in de afwezigheid van Hnf1α zijn samengevat in figuur 1. In wild-type muizen is LPH gen expressie het hoogste voor de geboorte en neemt af tijdens het zogen. In de afwezigheid van Hnf1α is LPH gen expressie voor de helft gereduceerd (~50%) in de dunne darm tijdens de vroege ontwikkeling, terwijl er na het zogen een bijna volledige uitschakeling is van LPH gen expressie. In tegenstelling tot deze bevindingen, heeft de inactivatie van Gata4, geen effect op LPH expressieniveaus tijdens de dunne darm ontwikkeling. Gezamenlijk laten deze data zien dat er een verschil is in de afhankelijkheid voor Gata4 en Hnf1α in de expressie van LPH tijdens de ontwikkeling van de dunne darm. Om na te gaan of dit verschil genspecifiek is, zijn de genexpressie niveaus van Fabp1 ook bepaald in de twee knockout modellen. De expressiepatronen van Fabp1 en LPH in de dunne darm komen sterk overeen, waardoor vergelijkbare regulatiemechanisme kunnen worden verwacht. Dit hoofdstuk laat echter zien dat in de dunne darm van de ontwikkelende knockout muizen *Fabp1* genexpressie verlaagd is (~50%) ten opzichte van wildtype muizen. Deze resultaten laten zien dat Gata4 en Hnf1α deels noodzakelijk zijn voor de expressie van LPH en Fabp1 voor het zogen, in tegenstelling tot de absolute onmisbaarheid van Gata4 en Hnf1α voor de genexpressie van LPH en Fabp1 na het zogen (zoals geconcludeerd in hoofdstuk 3 en 4). Om de rol van Hnf1α en Gata4 in de activatie van *SI* gen expressie tijdens het zogen zijn ook de SI niveaus bepaald in de twee knockout muismodellen. Zoals te zien is in figuur 1 zijn de SI gen

expressie niveaus in de knockout muizen niet te onderscheiden van de niveaus in wildtype muizen. Hiermee kan worden geconcludeerd dat Gata4 en Hnf1α niet noodzakelijk zijn voor de expressie van SI in deze ontwikkelingsfases.

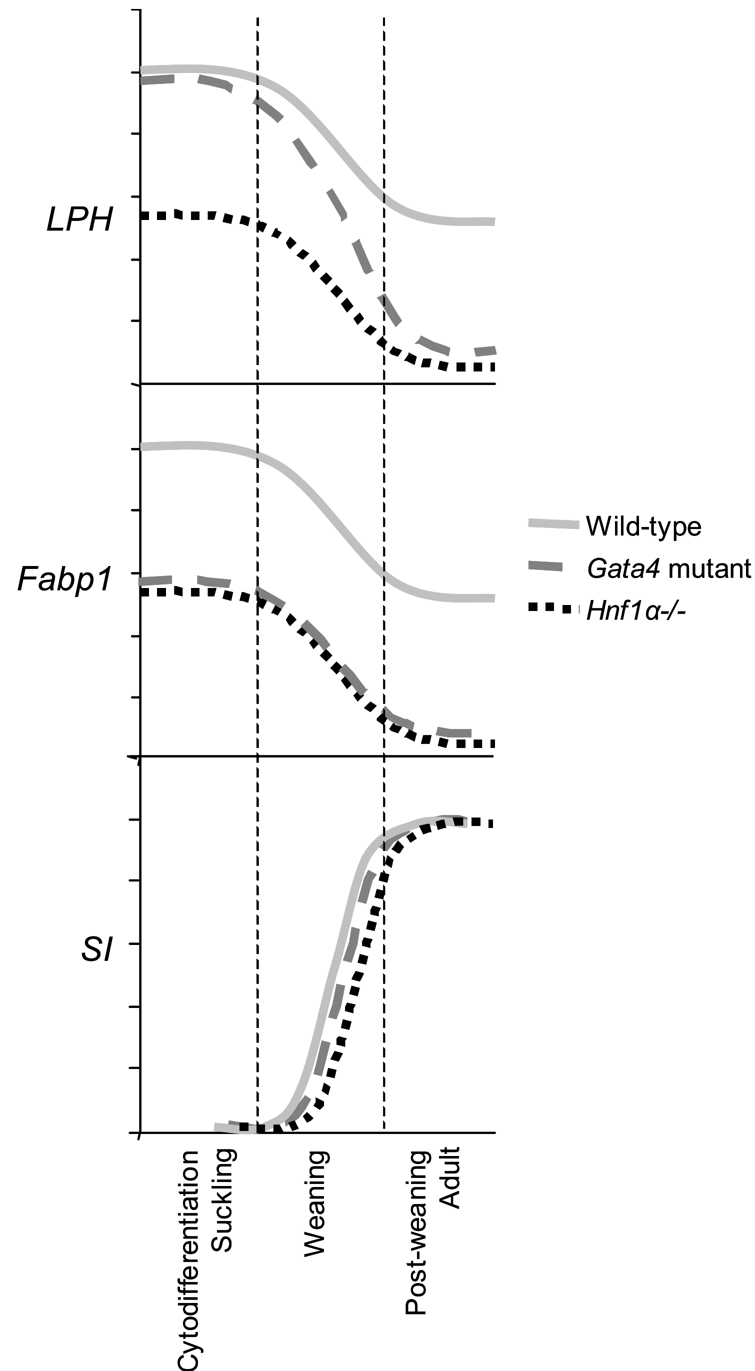

Fig. 1. Schematische weergave van de LPH, Fabp1 and SI genexpressieniveaus tijdens dunne darm ontwikkeling in wildtype, Gata4 mutant en Hnf1α-/- muizen. Schematische weergave van *LPH* (*top*), *Fabp1* (*midden*), en *SI* (*onder*) gen expressie in wildtype, Gata4 mutant, en Hnf1α-/- in de dunne darm tijdens de ontwikkeling.

Daarnaast wordt er in dit hoofdstuk de expressiepatronen van Gata4 en Hnf1α mRNA en eiwit tijdens de ontwikkeling van de dunne darm nauwkeurig bestudeerd. Tijdens de embryonale ontwikkeling nemen de expressieniveaus van Gata4 en Hnf1α toe en co-immunofluoresentie laat co-expressie zien van beide transcriptiefactoren in de nuclei van E16.5 epitheliale cellen. Na de geboorte, van P7 tot P14, is er een discordante relatie tussen de mRNA– en de nucleaire eiwit niveaus van Gata4 en Hnf1a in de dunne darm. In deze ontwikkelingsfase worden hoge mRNA niveaus gevonden in contrast met lage nucleaire eiwit niveaus. Samengenomen laat hoofdstuk 6 een verzameling data zien waaruit overtuigend blijkt dat de afhankelijkheid van Gata4 en Hnf1α voor de regulatie van genen in de dunne darm afhangt van de fase in de ontwikkeling. Alhoewel Gata4 en Hnf1α onmisbaar zijn voor de instandhouding van specifieke genen in de volwassen darm, deze transcriptiefactoren zijn maar deels van belang voor de expressie van dezelfde genen in de dunne darm van ontwikkelende muizen, waaruit blijkt dat andere regulatiemechanisme actief zijn in deze fases.

8.5 Interacties met FOG zijn noodzakelijk voor Gata4 repressie in de dunne darm.

In **hoofdstuk 6** wordt de rol van FOG co-factoren en Gata4 in het bepalen van de proximale expressiegrens van het ileum specifieke gen, *Asbt*, in de muis dunne darm, bestudeerd. Wij laten zien dat na de inactivatie van Gata4 tijdens het zogen, de horizontale expressie van Asbt zich uitstrekt tot het proximale jejunum, waaruit blijkt dat Gata4 cruciaal is voor het bepalen van de proximale Asbt expressiegrens. Verder laten we zien hoe de Gata4 afhankelijke repressie van *Asbt* expressie kan worden gerecapituleerd in CT-26 cellen, een cellijn geïsoleerd van muis adenocarcinomen uit het colon met kenmerken van de enterocyten in het ileum. Gata4 overexpressie in deze cellen resulteert in de repressie van een 1.1kb Asbt promoter-reporter plasmide, waarin 6 potentiële GATA bindingsplaatsen aanwezig zijn. Dit experiment toont aan dat het mechanisme van Gata4 repressie functioneert via de 1.1kb Asbt promoter sequentie.

Het is in andere orgaansystemen aangetoond dat belangrijke GATA functies afhankelijk zijn van de interactie met de FOG (Friend of GATA) co-factoren. Het doel van dit hoofdstuk is geweest om te bestuderen welke darm specifieke Gata4-functies afhankelijk zijn van de interactie met FOG co-factoren. In eerste instantie wordt de expressie van FOG co-factoren (Fog1 en Fog2) in dit hoofdstuk geanalyseerd in de volwassen muis dunne darm. Er is gevonden dat met name Fog1 sterk tot expressie komt in de dunne darm en dat het horizontale expressiepatroon overeenkomstig is met de Gata4 expressie, waarmee er een topografische basis is voor functionele interacties. Om de functie te testen is gebruik gemaakt van een *Gata4* knock-in muislijn waarin een mutant Gata4 tot expressie komt met een puntmutatie die leidt tot een enkele aminozuur vervanging in de N-terminale zinkvinger, die ervoor zorgt dat er geen binding kan plaatsvinden tussen Gata4 en FOG co-factoren. Wij laten zien hoe wij deze muislijn hebben geïntrigeerd met onze conditionele, induceerbare *Gata4* mutant om uiteindelijk een darmspecifieke, induceerbare *Gata4* knock-in muis te creëren. In deze *Gata4* knock-in muizen is

geen effect gezien op de hoeveelheid Goblet cellen, waaruit blijkt dat de rol van Gata4 in celcompositie onafhankelijk is van FOG co-factoren. Er is ook geen verschil gevonden in de expressie van *LPH*, waaruit blijkt dat ook deze Gata4-functie onafhankelijk is van FOG co-factoren. Daarentegen hebben de Gatat4 knock-in muizen wel een sterke toename in *Asbt* expressie in het jejunum. Samengenomen laten deze data zien, dat de Gata4 afhankelijke repressie van ileumgenen in het jejunum voor een deel tot stand komt uit de interactie met FOG co-factoren.

8.6 Een model voor regulatie in de dunne darm door Gata4 en Hnf1α in vivo.

Het gebruik van knock-out muismodellen voor zowel Gata4 als Hnf1α heeft de kennis en het begrip van de manier waarop deze transcriptiefactoren darmgenen reguleren vergroot. Uit deze studies kan een model worden samengesteld waarin Gata4 een centrale rol heeft in het dirigeren van positie-specifieke genexpressie door het aangaan van interacties met andere regulerende eiwitten, zoals Hnf1α en FOG co-factoren. In dit model speelt Gata4 een cruciale rol in drie verschillende regulatiemechanismen (Fig. 2).

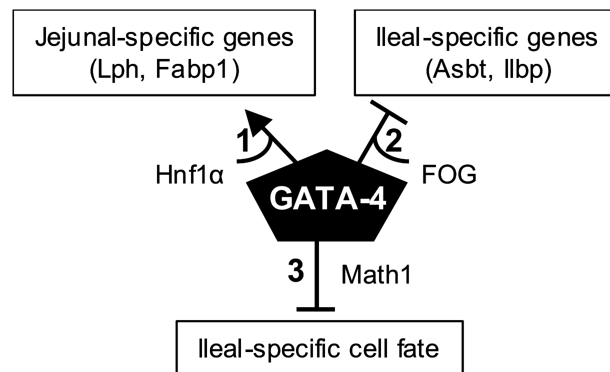

Fig. 2. Model waarin de drie Gata4-afhankelijke regulatiemechanismen zijn weergegeven. Onze hypothese is dat (1) Gata4 en Hnf1α gezamenlijk jejunum-specifieke genen activeren, (2) dat Gata4 met FOG co-factoren interacties aangaat om de expressie van ileum specifieke-genen te blokkeren en (3) dat Gata4 ileum specifieke cell differentiatie onderdrukt.

In het eerste regulatiemechanisme (*1*), in overeenstemming met de celkweek studies, zijn zowel Gata4 als Hnf1α noodzakelijk voor de activatie van jejunum-specifieke genen in de volwassen muis dunne darm (**hoofdstuk 3&4**). Onze hypothesis is daarom, op basis van de studies beschreven in **hoofstuk 2**, dat een fysieke interactie tussen Gata4 en Hnf1α en de proximale promoters noodzakelijk is voor de synergistische activatie van deze genen. In het tweede mechanisme (2), speelt Gata4 een cruciale rol in de onderdrukking van de expressie van ileum-specifieke genen in het jejunum (**hoofdstuk 4**). Onze hypothese, op basis van de experimenten beschreven in **hoofdstuk 6,** is dat deze Gata4-afhankelijke repressie tot stand komt uit interacties

met FOG co-factoren en waarschijnlijk ook door middel van interacties met de specifieke DNA sequenties van de promoters. In het laatste mechanisme (3) onderdrukt Gata4 de hoeveelheid Goblet cellen en onderhoudt het de distributie van enteroendocriene subpopulaties in het jejunum. Gezien de toegenomen expressie van *Math1* in de *Gata4* mutant muizen, is onze hypothese dat deze Gata4 functie verloopt via de onderdrukking van *Math1* expressie door middel van een nog onverklaarde weg. Een belangrijke kanttekening bij dit model komt aan de orde in **hoofdstuk 5,** waarin naar voren komt dat de afhankelijkheid van Gata4 en Hnf1α voor genregulatie pas intreedt na het zogen. Onze hypothesis is daarom dat voor en tijdens het zogen andere regulerende eiwitten verantwoordelijk zijn voor de expressie van bepaalde darmspecifieke genen. In het hoofdstuk "**7.2 future perspective**" worden experimenten beschreven, die zullen bijdragen aan een beter begrip van deze mechanismen en zullen leiden tot de ontdekking van nieuwe cruciale factoren die een rol spelen in de complexe regulatie van darmspecifieke genexpressie.

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To appreciate this work fully, it has to be viewed through its historical perspective. This thesis describes the latest work of the lactase research group that thanks its existence to a meeting of Professor dr. Hans Büller and Professor Richard Grand (MD) in a restaurant near Children's Hospital back in 1982. Some scribbling notes on a napkin were the start of a longstanding research collaboration that is still fully alive in 2006. In the past 24 years, over 60 Dutch students have spent 6 months, a year, or longer in the laboratory of Professor Grand in Boston, learning the basics of molecular biology and enjoying the beauty of Boston. Former PhD-students, such as dr. Edmond H.M.M. Rings and dr. Herbert M. van Wering, stayed longer and were crucial for the continuation of the lactase research. It was an honor for me to join the group in 2002, and to become part of this historic collaboration.

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CURRICULUM VITAE

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Abstracts:

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Digestive Disease Week 2003 (DDW): Orlando, Florida
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FASEB summer research conference 2005: Snowmass, Colorado
(Poster presentation)

Tjalling Bosse, John J. Fialkovich, Christina M. Piaseckyj, Eva Beuling, Robert K. Montgomery, Stephen D. Krasinski**.** Differential regulation of intestinal gene expression by Hnf1α during the weaning transition in mice.
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Tjalling Bosse, John J. Fialkovich, Christina M. Piaseckyj, Eva Beuling, Henrike Broekman, Robert K. Montgomery, Stephen D. Krasinski. Differential regulation of intestinal gene expression by Hnf1α during the weaning transition in mice.
Digestive Disease Week 2006 (DDW): L.A., Califonia
(Poster presentation)

Tjalling Bosse, Christina M. Piaseckyj, Ellen Burghard, John. J. Fialkovich, Satish Rajogal, William Pu, Stephen D. Krasinski. Gata4 is essential for the maintenance of jejunal-ileal identities in the adult mouse small intestine.
Digestive Disease Week 2006 (DDW): L.A., Califonia
(Oral presentation at the distinguished abstract plenary session)

Intestinal specific gene regulation by transcription factors Gata4 and Hnf1α in vivo

Tjalling Bosse

Stellingen:

1. Gata4 is the principal GATA member responsible for the expression of lactase-phlorizin hydrolase in the adult mouse intestine.

2. Hnf1α is indispensable for the regulation of a subset of intestinal genes, including lactase-phlorizin hydrolase.

3. Gata4 is an positional factor that is essential in the maintenance of jejunal-ileal identities in the adult small intestine.

4. Lactase-phlorizin hydorase is differentially regulated during weaning by Gata4 and Hnf1α in vivo.

5. Gata4 and Hnf1α are both dispensable for sucrase isomaltase gene expression in vivo.

6. The dimensions time and space determine transcription factor function as well as human behavior.

7. Bij het samenstellen van een proefschrift dient de verhouding tussen inspiratie en transpiratie 15% tot 85% te zijn (ongeveer van die orde). Is die verhouding andersom dan is er iets mis.
   dr Gerrit Wielenga, patholoog

8. Wetenschap probeert de zintuiglijk waarneembare wereld redelijk te verklaren, maar of de hele werkelijkheid zintuiglijk waarneembaar is en zich redelijk laat verklaren kan niet bewezen worden maar wordt geloofd.
   Mr. J.P.H. Donner, minister van Justitie

9. Gezien de internationalisering van de samenleving en het belang van holisme in de geneeskunde zou van de opleiding van iedere medicus zowel een verblijf in het buitenland als een stage op een afdeling Pathologie deel moeten uitmaken.
   Prof. dr J.A. Bruijn, patholoog

10. Orthopeden zijn het vaker met zich zelf eens dan met elkaar.

11. Koken wordt door velen als kunst beschouwd omdat het creativiteit en vaardigheid vereist. Dezelfde eisen gelden voor het beoefenen van de wetenschap.