Published in final edited form as:
Dev Biol. 2008 October 1; 322(1): 179–189. doi:10.1016/j.ydbio.2008.07.022.

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

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Abstract

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

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Keywords

GATA4; Friend of GATA; FOG; intestinal differentiation; apical sodium-dependent bile acid transporter; ASBT

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 (Gordon et al., 1992). This functional diversity is linked to a continuous renewal process in which stem cells located at or near the base of crypts produce transit amplifying cells that ultimately differentiate into four principal cell types. Absorptive enterocytes, which constitute the majority of intestinal epithelial cells, enteroendocrine cells, and goblet cells migrate up the villi and are shed into the intestinal lumen every three to five days, whereas Paneth cells reside at the base of crypts and turn over at a slower rate. Specific absorptive enterocyte genes that encode proteins that mediate absorption of bile salts are localized to the distal ileum, including the apical sodium-dependent bile acid transporter (ASBT) (Shneider, 2001) and ileal lipid binding protein (ILBP) (Crossman et al., 1994). Proteins responsible for the terminal digestion and absorption of most nutrients are localized to jejunum and proximal ileum, as exemplified by lactase-phlorizin hydrolase (LPH) (Krasinski et al., 1997) and liver fatty acid binding protein (FABP1) (Simon et al., 1993). Goblet cells are more numerous in distal small intestine (Specian and Oliver, 1991), and enteroendocrine subpopulations display a functional diversity characterized by the regional segregation of hormones that activate (e.g. cholecystokinin, CCK) or repress (e.g. peptide YY, PYY) gastrointestinal processes (Schonhoff et al., 2004). Maintenance of a dynamic diversity in gene expression and cell fate allocation along the cephalo-caudal axis is necessary for the normal functioning of the small intestine.

Recently, we found that GATA4 is a key regulator of regional gene expression and cell fate allocation in the adult mouse small intestine (Bosse et al., 2006a). GATA4 is a member of a conserved family of transcription factors that contain a pair of zinc fingers that mediate binding to their consensus DNA sequence, WGATAR, in the regulatory region of target genes (Molkentin, 2000). In the small intestine of adult rodents and humans, GATA4 is expressed at high levels in proximal regions, but is undetectable in distal ileum (Bosse et al., 2006a; van Wering et al., 2004). Conditional, inducible inactivation of *Gata4* in the adult mouse jejunum results in a generalized transformation to an ileal-like phenotype that is characterized by an induction of ileal-specific genes, including *Asbt* and *Ilbp*, and attenuation of genes restricted to proximal small intestine, including *Lph* and *Fabp1*. Furthermore, secretory cell fate also becomes ileal-like as indicated by a significant increase in goblet cell number and the abundance of the mRNA for *Math1*, a secretory cell mediator (Yang et al., 2001), and a trend toward an increase in *Pyy* mRNA and a decrease in *Cck* mRNA (Bosse et al., 2006a). This novel finding establishes a fundamental plasticity in the adult mammalian small intestine not previously realized, and highlights multiple levels of regulation by GATA4 in this organ involving gene repression, gene activation, and cell fate determination. However, the precise mechanisms by which GATA4 regulates these diverse functions in the mature small intestine are currently unknown.

Friend of GATA (FOG) is an evolutionarily conserved multi-zinc finger cofactor family whose members physically associate with GATA factors, and mediate GATA function in a broad array of tissues and cell types (Cantor and Orkin, 2005). The GATA-FOG interaction is conserved in *Drosophila* where the FOG homolog, *U-shaped*, physically associates with *pannier*, a GATA homolog (Haenlin et al., 1997), indicating the fundamental importance of

this interaction. Using a split two-hybrid screen, a GATA1 mutant (GATA1ki) with a valine-to-glycine substitution at position 205 in the N-terminal zinc finger was identified that has attenuated binding affinity for FOG cofactors, but normal DNA binding function (Crispino et al., 1999). *Gata1*$^{ki/ki}$ mice that express GATA1ki in place of GATA1 die during embryogenesis due to anemia caused by disrupted erythroid maturation and megakaryocyte abnormalities (Crispino et al., 1999). This phenotype is similar to that in *Gata1*$^{-/-}$ (Chang et al., 2002; Fujiwara et al., 1996; Pevny et al., 1991) or *Fog1*$^{-/-}$ (Tsang et al., 1998) mice, indicating that GATA1-FOG1 interaction is required for hematopoiesis. A FOG1 mutant that restores GATA1-FOG1 interaction rescues this phenotype, providing in vivo evidence that the *Gata1* knock-in mutation specifically disrupts the ability of GATA1 to bind FOG cofactors, and that GATA1ki is otherwise functional (Cantor et al., 2002; Crispino et al., 1999). In addition, a *Gata4* knock-in model was designed with the analogous valine-to-glycine substitution at position 217 in GATA4 (GATA4ki) that disrupts the interaction between GATA4 and FOG cofactors (Crispino et al., 2001; Tevosian et al., 2002). *Gata4*$^{ki/ki}$ mice show an embryonic lethal cardiac phenotype, very similar to that found in *Gata4*$^{-/-}$ (Kuo et al., 1997; Molkentin et al., 1997) and *Fog2*$^{-/-}$ (Svensson et al., 2000; Tevosian et al., 2000) mice, providing evidence that GATA4-FOG2 interaction is required for cardiogenesis. Furthermore, the *Gata4*^{ki} model has revealed that GATA4-FOG interaction is important in gonadal differentiation (Manuylov et al., 2007; Tevosian et al., 2002) and gastric epithelial development (Jacobsen et al., 2005).

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

MATERIALS AND METHODS

Mice

Mice were housed under standard conditions in the Animal Research at Children's Hospital (ARCH) facility and provided food and water ad libitum. Approval was obtained from the Institutional Animal Care and Use Committee. Four *Gata4* alleles were utilized in this study (Fig. 1A). The *Gata4*^{flox} allele, which contains loxP sites flanking the translational start site and the region encoding the activation domains of GATA4, expresses wild-type GATA4 (Pu et al., 2004). Exposure of *Gata4*^{flox} to the recombinase CRE results in exon2 excision (*Gata4*$^{\Delta ex2}$), and the subsequent utilization of an alternative in-frame ATG in exon3 leading to the synthesis of a truncated, transcriptionally inactive form of GATA4 (GATA4Δex2) devoid of its activation domains (Bosse et al., 2006a). The *Gata4*^{ki} allele encodes a mutant GATA4 (GATA4ki) that contains a single amino acid substitution (V217G) in the N-terminal zinc finger rendering it unable to bind specifically with FOG cofactors (Crispino et al., 2001). The wild-type allele (*Gata4*^{wt}), was also utilized in the study. Genotyping to distinguish among each of the four *Gata4* alleles was conducted by PCR on DNA extracted from tail biopsies as described (Bosse et al., 2006b) using primers specific for exon2 or exon5 of *Gata4* (see supplemental Fig. 1 (Fig. S1)).

Two different conditional, inducible genetic mouse models were used for this study, including a previously validated conditional *Gata4* inactivation model (Bosse et al., 2006a),

and a novel conditional *Gata4* knock-in model (Fig. 1B). Both models were established in a Villin*Cre*ER^{T2} transgenic background (el Marjou et al., 2004) in which CRE-mediated excision of floxed *Gata4* DNA occurs specifically in intestinal and colonic epithelium after tamoxifen treatment (Bosse et al., 2006a). Adult mice (6–8 weeks) were treated with five single intraperitoneal injections of tamoxifen (100μl, 10mg/ml) per day for 5 consecutive days and sacrificed for tissue collection 14 days after the last injection (Bosse et al., 2006a), unless indicated otherwise. In the *Gata4* inactivation model (Fig. 1B, Model I), *Gata4*$^{flox/flox}$, Villin*Cre*ER^{T2}-positive mice were treated with tamoxifen resulting in exon2 deletion (*Gata4*$^{\Delta ex2}$) and subsequent conditional *Gata4* inactivation in the intestinal epithelium (Bosse et al., 2006a). *Gata4*$^{flox/flox}$, Villin*Cre*ER^{T2}-negative mice treated with tamoxifen were used as controls. In the *Gata4* knock-in model (Fig. 1B, Model II), *Gata4*$^{ki/flox}$ knock-in mice and *Gata4*$^{wt/flox}$ controls (het-controls) were established in a Villin*Cre*ER^{T2} background. Before tamoxifen treatment, both knock-in mice and het-controls produce wild-type GATA4 from at least one allele in all GATA4 expressing tissue, including the small intestine. After tamoxifen treatment of knock-in mice, GATA4 and GATA4ki are expressed in all tissues that normally express GATA4, except in the intestinal epithelium, where GATA4 from the *Gata4*^{flox} allele is specifically inactivated by CRE-mediated excision, but GATA4ki continues to be expressed from the *Gata4*^{ki} allele. After tamoxifen treatment of het-controls, GATA4 is expressed from both alleles in all tissues that normally express GATA4, except in the intestinal epithelium, where *Gata4*^{flox} is excised by CRE, but GATA4 continues to be expressed from *Gata4*^{wt} allele. This approach creates a conditional, inducible *Gata4* knock-in model.

RNA isolation

RNA was isolated from heart, stomach, and small intestine using the RNeasy kit (Qiagen) as described previously (Bosse et al., 2006a). Intestinal segments (0.5 to 1.0 cm) were obtained from the most proximal region adjacent to the pylorus (segment 1), the 25% mark (segment 2), the geometric center (segment 3), the 75% mark (segment 4), and the most distal region adjacent to the ileocecal junction (segment 5).

Sequencing

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

RT-PCR

To quantify mRNA abundances, semi-quantitative and real-time RT-PCR were conducted as described previously (Bosse et al., 2006a). Primer pairs (Fig. S2) were designed using Beacon Design software (Biosoft International) and optimized. Real-time RT-PCR was carried out using an iCycler and iQ SYBR Green Supermix (Bio-Rad). *Gapdh* mRNA abundance was measured for each sample and used to normalize the data. All data were expressed relative to a calibrator as indicated in the figure legends.

Immunoblotting

Crude nuclear extracts were isolated from four quarters of intestine (proximal to distal: I – IV) and Western analysis was conducted as described previously (van Wering et al., 2004) using 100 μg of extract. The membranes were blocked for 1 h at room temperature in 5%

nonfat dried milk in PBS and incubated with goat anti-FOG1 (1:1500, Santa Cruz) with or without FOG1 blocking peptide (1:1500, Santa Cruz) for 1 h. Membranes were stripped and re-probed using mouse anti-β-actin (1:4000, Santa Cruz). Horseradish peroxidase-linked secondary antibodies and chemiluminescence solution (Pierce West Femto Kit) were used to visualize FOG1 or β-actin signals.

In situ hybridization

RNA probes were prepared by in vitro transcription of a partial cDNA insert from the *Fog1* library plasmid M10 subcloned into pBluescript KS (Stratagene) (Tsang et al., 1997) using digoxigenin-UTP (Roche Molecular Biochemicals), and T3 (antisense) or T7 (sense) polymerase as described (Katz et al., 2003). In situ hybridization assays were conducted as described previously (Tevosian et al., 2000).

Immunohistochemistry

Intestinal segments were fixed in ice-cold 4% paraformaldehyde in PBS for 4 h, dehydrated overnight as described (Bosse et al., 2006a), embedded in paraffin, and sectioned (5 μm) in the Department of Pathology at Children's Hospital Boston. After deparaffinization and antigen retrieval (Bosse et al., 2006a; Bosse et al., 2006b), the sections were incubated with the primary antibody for 1 h at 37°C, rinsed, and then incubated with the secondary antibody for 1 h at 37°C. For immunofluorescence, sections were incubated in a solution containing 4',6-diamino-2-phenylindol dihydrochloride (DAPI, 2 μg/ml, Molecular Probes) in PBS for 15 min at room temperature, washed in PBS, and mounted in Mowiol mounting medium (Calbiochem). For immunohistochemistry, biotinylated secondary antibodies were linked to avidin-horseradish peroxidase or avidin-alkaline phosphatase conjugates (Vector Labs), and visualized using 3,3'-diamino benzidine (DAB) for 2–5 min or 4-nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3indolyl-phosphate (BCIP) for 20–90 min, respectively. For selected sections, the tissue was lightly counterstained with methyl green.

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

Statistical analyses

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

RESULTS

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

As previously shown (Bosse et al., 2006a), conditional, inducible *Gata4* inactivation by excision of the activation domains of GATA4 (synthesis of GATA4Δex2), results in an induction in jejunum of absorptive enterocyte genes normally restricted to distal ileum, including *Asbt* and *Ilbp*. Since GATA4 is expressed in both crypt and villus epithelial cells (Bosse et al., 2006a; Divine et al., 2004; Dusing and Wiginton, 2005; van Wering et al.,

2004), it is uncertain whether GATA4 represses ileal-specific genes in proximal small intestine by a process that occurs in differentiated absorptive enterocytes on villi, and/or is determined early in the differentiation program in crypt progenitor cells. To localize the site of action for GATA4-mediated repression of intestinal genes, the induction of *Asbt* expression during the initial phases of *Gata4* inactivation was characterized. *Gata4*$^{flox/flox}$ mice positive for the Villin*Cre*ER^{T2} transgene were treated for one day or two days with tamoxifen, and sacrificed 24 h after the last injection. In mid-jejunum, CRE-mediated excision of *Gata4* was complete after only one injection of tamoxifen (Fig. 2A), and *Asbt* mRNA was induced (Fig. 2B). Because of the 3-day crypt-to-villus tip cell migration time in mice (Gordon et al., 1992), we hypothesized that if *Asbt* was induced directly in differentiated absorptive enterocytes, then ASBT would first be detected in a random, patchy pattern throughout the villi. Conversely, if *Asbt* was induced by a process that originated in crypts, then it would first appear in lower villi and migrate up the villi over time. ASBT, not normally present in mid-jejunum (Fig. 2C) (Dawson et al., 2003; Shneider et al., 1995), was first detected in the microvillus membrane of single cells scattered throughout the villi after one day of *Gata4* inactivation (Fig. 2D–F), and was increasingly expressed in a patchy pattern along the length of the villi extending to the villus tip after two days of *Gata4* inactivation (Fig. 2G–I). ASBT induction in the mid-jejunum of *Gata4* mutant mice is shown as a control (Fig. 2J). Although the design and outcome of this experiment cannot rule out the possibility that a component of GATA4-mediated *Asbt* repression in the jejunum is directed by GATA4 in crypt cells, these data are consistent with the hypothesis that GATA4 mediates *Asbt* repression in differentiated absorptive enterocytes on villi.

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

FOG cofactors mediate GATA-regulated repression of specific genes in multiple non-intestinal systems (Crispino et al., 1999; Grass et al., 2003; Hong et al., 2005; Letting et al., 2004; Lu et al., 1999; Roche et al., 2008; Svensson et al., 1999; Tsang et al., 1997), but their function and expression in the small intestine is unknown. To test the hypothesis that FOG cofactors mediate the GATA4-regulated repression of ileal-specific absorptive enterocyte genes in the proximal small intestine, we established *Gata4*$^{ki/flox}$, Villin*Cre*ER^{T2}-positive (knock-in) mice, and *Gata4*$^{wt/flox}$, Villin*Cre*ER^{T2}-positive heterozygous controls (het-controls) (Fig. 1). All genotypes were confirmed by PCR using primer pairs that distinguish the Gata*4*^{ki} from the *Gata4*^{flox} or *Gata4*^{wt} alleles, as well as the *Gata4*^{flox} from the *Gata4*^{ki} or *Gata4*^{wt} alleles (Fig. S3A).

To validate intestine-specific CRE-mediated excision of the *Gata4*^{flox} alleles, and specific allelic expression from the *Gata4*^{ki} allele in the knock-in mice, and from the *Gata4*^{wt} allele in the het-controls, RT-PCR and cDNA sequencing was conducted on RNA samples from mid-jejunum. The *Gata4*^{flox} allele, common to both knock-in and het-control mice, was excised in jejunum but not heart after tamoxifen treatment (Fig. S3B), verifying intestine-specific CRE-mediated excision. To distinguish expression from the *Gata4*^{ki} vs. *Gata4*^{wt} alleles, the region encompassing the V217G knock-in mutation was amplified by RT-PCR using the Ki3 and Ki4 primers (Fig. S3C). Because Ki3 hybridizes to exon2 sequence, and because this exon is excised from the *Gata4*^{flox} allele after tamoxifen treatment producing the recombined *Gata4*$^{\Delta ex2}$ allele, the *Gata4Δex2* cDNA is not amplified. Using a nested primer (Ki5), the knock-in or wild-type cDNA was then confirmed by sequencing (Fig. S3C). For all mice in this study, there was no evidence of band ambiguity at the knock-in site for *Gata4* knock-in mice, verifying that CRE-mediated excision was complete.

The expression of *Gata4*, *Fog1*, and *Fog2* in the knock-in mice and het-controls was determined (Fig. 3). Because the *Gata4*^{ki}, *Gata4*^{flox}, and *Gata4*^{wt} alleles are all under the

control of the endogenous *Gata4* promoter, we quantified total *Gata4* mRNA abundance as the most sensitive indicator of a possible knock-in effect on *Gata4* expression. Total *Gata4* and *Fog1* mRNA abundances were not significantly different between the two groups, but *Fog2* mRNA was significantly lower (60%, $P<0.05$) in the knock-in mice as compared to the het-controls. Noteworthy, the cycle threshold (C_T) for *Fog1* was ~9 cycles lower as compared to *Fog2* indicating that *Fog1* mRNA abundance is 1000-fold higher than that of *Fog2*. These data suggest that although *Fog2* mRNA is expressed at low levels, its abundance is dependent on GATA4-FOG interactions.

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

To test the hypothesis that FOG cofactors specifically mediate GATA4-regulated repression of ileal-specific absorptive enterocyte genes in proximal small intestine, the jejunal expression of absorptive enterocyte target genes and lineage markers were compared between *Gata4* knock-in mice and het-controls. As shown in Fig. 4, the mRNA abundances of *Asbt* and *Ilbp* (Fig. 4A) in jejunum of the knock-in mice were induced 4-fold ($P<0.01$) and 14-fold ($P<0.001$), respectively, compared to the het-controls, consistent with a requirement of GATA4-FOG interactions for repression of ileal-specific genes in the jejunum. Noteworthy, *Asbt* and *Ilbp* mRNAs, which are known to be undetectable in wild-type mid-jejunum (Bosse et al., 2006a;Dawson et al., 2003;Sacchettini et al., 1990;Shneider et al., 1995), were induced in the jejunum of the het-control mice, indicating either a heterozygous effect, or a dominant negative effect of GATA4Δex2 on wild-type GATA4, suggesting that repression of *Asbt* and *Ilbp* by GATA4 is dose-dependent in vivo. ASBT was highly induced on the microvillus membrane of villus enterocytes in the *Gata4* knock-in mice (Fig. 4B), and was detected in isolated cells in the het-controls (Fig. 4C), generally consistent with *Asbt* mRNA abundances in the two groups. Undetectable expression in wild-type jejunum (Fig. 4D) and endogenous expression in wild-type ileum (Fig. 4E) are shown for reference.

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

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

To begin to elucidate which FOG cofactor may be responsible for mediating GATA4 function, the expression patterns of *Fog1* and *Fog2* were determined in the adult mouse small intestine. *Fog1* mRNA abundance demonstrated a decreasing proximal-to-distal pattern (Fig. 6A, upper) similar to that of *Gata4* (Bosse et al., 2006a;van Wering et al., 2004). The abundance of *Fog1* mRNA in proximal duodenum (segment 1) was similar to that in stomach, but in distal ileum (segment 5) was ~10% of that in stomach, and similar to the low level of expression in heart. *Fog2* mRNA was low proximally and increased distally (Fig. 6A, lower), a pattern reciprocal to that of *Gata4* and *Fog1*. Intestinal *Fog2* mRNA abundance was similar or less than that in stomach, and ranged from 7–20% of that in heart.

To determine whether FOG proteins mimic their mRNA distributions, Western blot analyses were carried out. Since *Fog2* mRNA abundance was low in the small intestine (~1000-fold less than *Fog1*), and FOG2 protein could not be detected by immunohistochemistry (data not shown), only protein patterns for FOG1 were analyzed. As shown in Fig. 6B, a 160 kDa band corresponding to FOG1 (Cantor et al., 2002) demonstrated a declining proximal-to-distal pattern similar to that of its mRNA profile. The 160 kDa band, as well as bands with

faster mobilities, were specifically blocked by an epitope-specific polypeptide, verifying specific antigen detection. Taken together, these data demonstrate that FOG1 has a declining proximal-to-distal expression pattern in the adult small intestine that is similar to that of GATA4.

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

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

To delineate the cellular expression patterns of FOG1 in the small intestine, and its co-localization with GATA4, immunostaining for FOG1 and GATA4, along with lineage-specific markers, was conducted on proximal jejunum of adult mouse small intestine. We had previously demonstrated that GATA4 is expressed in absorptive enterocytes on villi, and in proliferating and lysozyme-positive crypt cells, but is not expressed in goblet or enteroendocrine cells (Bosse et al., 2006a). Using an alkaline phosphatase substrate detection approach, FOG1 was identified in the nuclei of epithelial cells throughout the crypts and villi (Fig. 8A), and serial section immunostaining revealed co-localization with GATA4 (Fig. 8B) in both compartments. FOG1 immunostaining could not be detected in the nuclei of goblet cells (Fig. 8C–E), as with GATA4 (Bosse et al., 2006a), but FOG1 immunostaining was detected in the nuclei of chromograninA-positive enteroendocrine cells (Fig. 8F–H), contrasting with the absence of GATA4 immunostaining in this lineage (Fig. 8I–K) (Bosse et al., 2006a). FOG1 immunostaining was also detected in lysozyme-positive (Fig. 8L,M) and proliferating (Fig. 8N,O) crypt cells, and is thus co-localized with GATA4 in this compartment (Bosse et al., 2006a). Taken together, these data show that FOG1 and GATA4 are co-localized in absorptive enterocytes on villi, and in lysozyme-positive and proliferating crypt cells of the adult mouse small intestine, demonstrating a topographic basis for possible interactions. These data also reveal divergent expression in chromagraninA-positive enteroendocrine cells, suggesting that FOG1 has functions in this lineage that are independent of GATA4.

DISCUSSION

GATA4, a transcription factor expressed in the proximal small intestine but not in the distal ileum, maintains proximal-distal distinctions in the mature small intestine by multiple processes involving gene repression, gene activation, and cell fate determination (Bosse et al., 2006a). Since interactions between GATA factors and FOG cofactors result in the repression of GATA-mediated transcriptional activation of hematopoietic and cardiac target promoters (Crispino et al., 1999; Grass et al., 2003; Hong et al., 2005; Letting et al., 2004; Lu et al., 1999; Roche et al., 2008; Svensson et al., 1999; Tsang et al., 1997), we tested the hypothesis that FOG cofactors are necessary for GATA4-mediated repression of ileal-specific absorptive enterocyte genes in the small intestine in vivo. We engineered a novel, inducible, intestine-specific *Gata4* knock-in model (Fig. 1), in which wild-type GATA4 is specifically inactivated in the intestine, but a GATA4 mutant that does not bind FOG cofactors (GATA4ki) continues to be expressed. We found that ileal-specific genes are induced in the proximal small intestine (Fig. 4), whereas genes restricted to proximal small intestine and cell lineage markers are unaffected (Fig. 5), indicating that GATA4-FOG

interactions contribute specifically to the repression function of GATA4 within this organ. Furthermore, co-expression of GATA4 and FOG1 in the nuclei of absorptive enterocytes on villi, and throughout the crypt epithelium (Fig. 8), suggests that FOG1 mediates GATA4-regulated repression of intestinal genes, although a role for FOG2 in this process cannot be discounted. These findings provide the first indication of FOG function and expression in the mammalian small intestine.

Repression of specific genes by GATA factors is well documented (Crispino et al., 1999; Grass et al., 2003; Letting et al., 2004; Lu et al., 1999; Svensson et al., 1999; Tsang et al., 1997), and generally occurs by recruitment of FOG cofactors to target gene promoters, which, in turn, mediates recruitment of the nucleosome remodelling and histone deacetylase (NuRD) complex leading to the deacetylation of local histones and gene silencing (Hong et al., 2005; Roche et al., 2008). Our data show that, GATA4 and FOG cofactors, specifically FOG1, are co-expressed in differentiated absorptive enterocytes on villi (Fig. 8), and are consistent with the hypothesis that GATA4 mediates *Asbt* repression in differentiated absorptive enterocytes on villi rather than by an upstream process dictated earlier in the differentiation process in crypt progenitor cells (Fig. 2). These findings have led us to hypothesize that GATA4 mediates *Asbt* repression by binding directly to the *Asbt* promoter. While it is possible that an indirect pathway within absorptive enterocytes, such as repression of another activator, or activation of a repressor, could mediate *Asbt* repression, we have previously shown that the mRNAs for known activators of *Asbt*, including hepatocyte nuclear factor-alpha (HNF1α) (Shih et al., 2001), liver receptor homolog-1 (LRH1) (Chen et al., 2003), and c-FOS (Chen et al., 2001), are not decreased in our conditional *Gata4* inactivation model (Bosse et al., 2006a). Thus, we hypothesize that GATA4-regulated repression of specific intestinal genes is mediated by promoter-dependent recruitment of GATA4-FOG complexes that may, in turn, promote histone deacetylation and gene silencing by recruitment of the NuRD complex. This model assumes that GATA4-FOG repression in the proximal small intestine hierarchically overrides activation by HNF1α, LRH1, c-FOS, or any other as yet unknown activator of *Asbt* gene expression.

Our data show that while the gene repression pathway is dependent on GATA4-FOG interactions (Fig. 4), the gene activation pathway is independent of this interaction (Fig. 5). We and others have previously shown using in vitro and cell culture models that GATA4 physically associates with HNF1α and synergistically activates the *Lph* and *Fabp1* promoters (Divine et al., 2004;van Wering et al., 2004) through an evolutionarily conserved mechanism (van Wering et al., 2002). Furthermore, both genes are strongly attenuated in intestine of mice in which *Gata4* is conditionally inactivated (Bosse et al., 2007;Bosse et al., 2006a), or *Hnf1α* is knocked out (Bosse et al., 2007;Bosse et al., 2006b). These findings support our original hypothesis that the overlapping expression of GATA4 and HNF1α in the intestinal epithelium, combined with specific promoter signatures in target genes, results in the activation of a specific subset of genes in proximal small intestine (Krasinski et al., 2001;van Wering et al., 2004;van Wering et al., 2002). It is intriguing to speculate that the promoter configurations in genes activated by GATA4-HNF1α cooperativity specifically exclude the recruitment of GATA4-FOG complexes.

We had previously shown that conditional inactivation of *Gata4* results in a significant increase in goblet cell number in the jejunum, and an increase in the expression of *Math1* (Bosse et al., 2006a), a mediator of secretory lineages in the intestine (Yang et al., 2001), consistent with a jejunum-to-ileum transformation in cell fate allocation. We also previously found in the jejunum a trend toward an increase in the mRNA abundance of *Muc2*, a goblet cell marker (Specian and Oliver, 1991), and a trend toward a decease in the mRNA for *Cck* and increase in that for *Pyy*, both of which are enteroendocrine cell markers that normally show an increasing and decreasing proximal-distal gradient (Schonhoff et al., 2004),

respectively. In the present study, we found no evidence that FOG cofactors are required for GATA4-regulated cell fate allocation in the intestine (Fig. 5). However, it should be noted that jejuno-ileal differences in cell lineage composition are subtle, and that these differences may be obscured by a gene dosage effect due to the heterozygous nature of the controls, and thus a role for GATA4-FOG interactions in these processes cannot be ruled out.

Both *Fog1* and *Fog2* mRNAs are expressed in the adult mouse small intestine, but *Fog1* is more abundant (~1000-fold), and demonstrates a quantitative pattern that is similar to that of *Gata4* (Fig. 6). In humans, *Fog1* mRNA is expressed in the proximal small intestine (Freson et al., 2003), whereas *Fog2* mRNA can not be detected (Holmes et al., 1999), supporting the relative differential abundances of *Fog1* and *Fog2* mRNAs in the mouse small intestine. FOG1 and GATA4 are co-expressed in absorptive enterocytes on villi, and throughout the proliferating and lysozyme-positive Paneth cells in crypts (Fig. 8), suggesting that FOG1 mediates GATA4 function. While FOG1 is generally associated with GATA1, and FOG2 with GATA4, FOG1 was shown to be necessary for heart development (Katz et al., 2003) presumably reflecting cooperation with the GATA4/5/6 subfamily. FOG1 is also expressed in the chromagraninA-positive enteroendocrine cells while GATA4 is not expressed in these cells (Bosse et al., 2006a), suggesting that FOG1 has functions in these cells that are independent of GATA4, possibly through interaction with GATA6, which is expressed in this lineage (Dusing and Wiginton, 2005). *Fog2* mRNA is detectible in the adult mouse small intestine (Fig. 6A), and is decreased in the *Gata4* knock-in mice as compared to the het-controls (Fig. 3), suggesting that its levels are regulated by GATA4-FOG interactions. Although our data support a role for FOG1 in mediating GATA4 function, we cannot rule out the possibility that FOG2, or both FOG1 and FOG2, are required for GATA4 function in the intestine. Correlation of our knock-in results with those of conditional *Fog1* and *Fog2* single and double knockout will allow a precise determination of FOG1 and FOG2 requirements for GATA4 function in this organ.

The demonstration of a GATA4-FOG requirement for gene expression in the small intestine adds to the growing list of tissues and cell types that require GATA-FOG interactions for normal function. In addition to the well documented role of GATA1-FOG1 interactions in hematopoiesis (Cantor and Orkin, 2005), GATA4-FOG interaction is required for coronary vasculature initiation, cardiac morphogenesis and valve formation during cardiogenesis (Crispino et al., 2001), differentiation of precursors into XY-specific Sertoli cells during gonad development (Tevosian et al., 2002), and epithelial-mesenchymal signaling during stomach development (Jacobsen et al., 2005). While these studies all highlight the critical roles of GATA4-FOG interactions for specific aspects of embryonic development, our study, due to the inducible nature of our model, is the first to demonstrate a role for GATA4-FOG interactions in adult intestine. Taken together, these studies underscore the diverse nature of GATA4-FOG functions, including morphogenesis, cell fate determination, and maintenance of terminal differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. W. T. Pu (Children's Hospital Boston, Boston, MA) for the floxed *Gata4* mouse line, S. Robine (Institut Curie, Paris, France) for the Villin*Cre*ER^{T2} transgenic mouse line, and P.A. Dawson (Wake Forest School of Medicine, Winson-Salem, NC) for the ASBT antibody. We also thank Lena Liu (Department of Pathology, Children's Hospital Boston, Boston, MA) for technical support.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R01-DK-061382 (SDK) and R37-DK-32658 (RJG), the Harvard Digestive Disease Center (5P30-DK-34854), and the Nutricia Research Foundation (EB and TB), the Foundation De Drie Lichten (EB) and the Foundation Doctor Catharine van Tussenbroek (EB) in The Netherlands.

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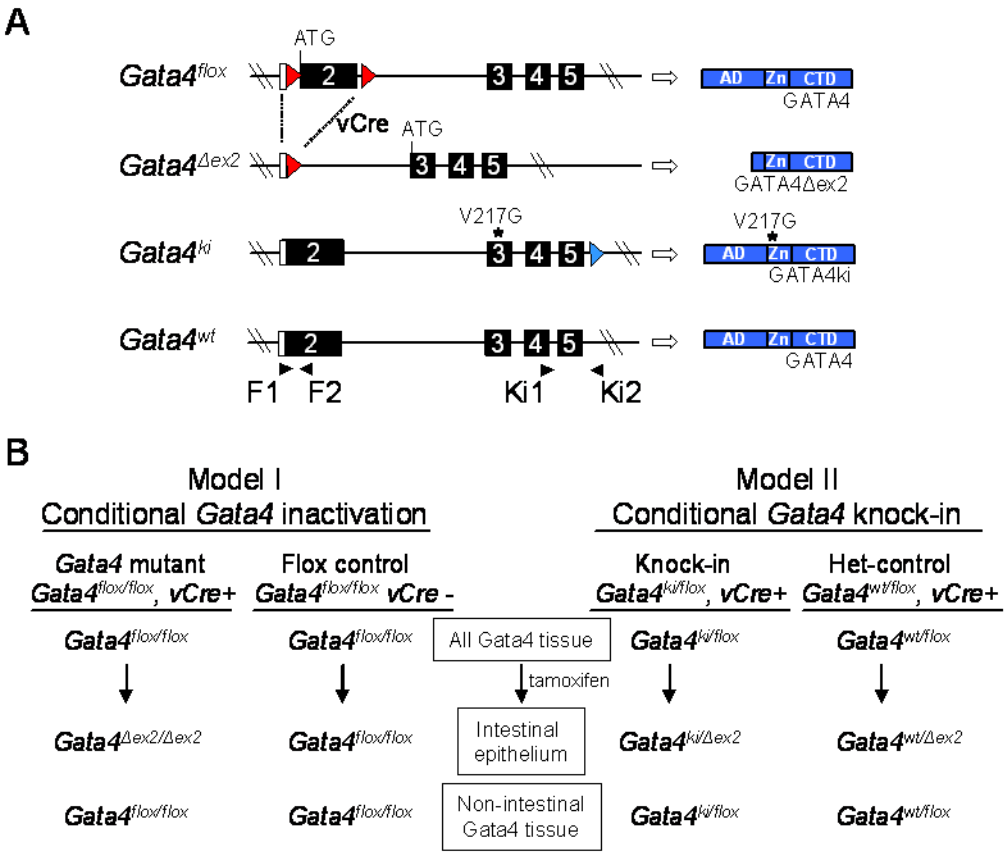

Fig. 1.

In vivo mouse models. (A) Schematic representation of the different *Gata4* alleles used in this study, and their protein products. Open boxes indicate untranslated region. Filled boxes indicate translated region. Numbers indicate exons. Red arrowheads indicate loxP sites used for excision of the GATA4 activation domains in the *Gata4*^{flox} allele. Blue arrowhead indicates a residual loxP site previously used to remove a neomycin cassette from the *Gata4*^{ki} allele (Crispino et al., 2001). Blue boxes indicate protein product. AD, activation domain; Zn, zinc fingers; CTD, C-terminal domain. Filled arrowheads indicate PCR primers. (B) Models used in this study showing the *Gata4* alleles in different tissue in test and control mice before and after tamoxifen treatment. vCre indicates the Villin*Cre*ER^{T2} transgene.

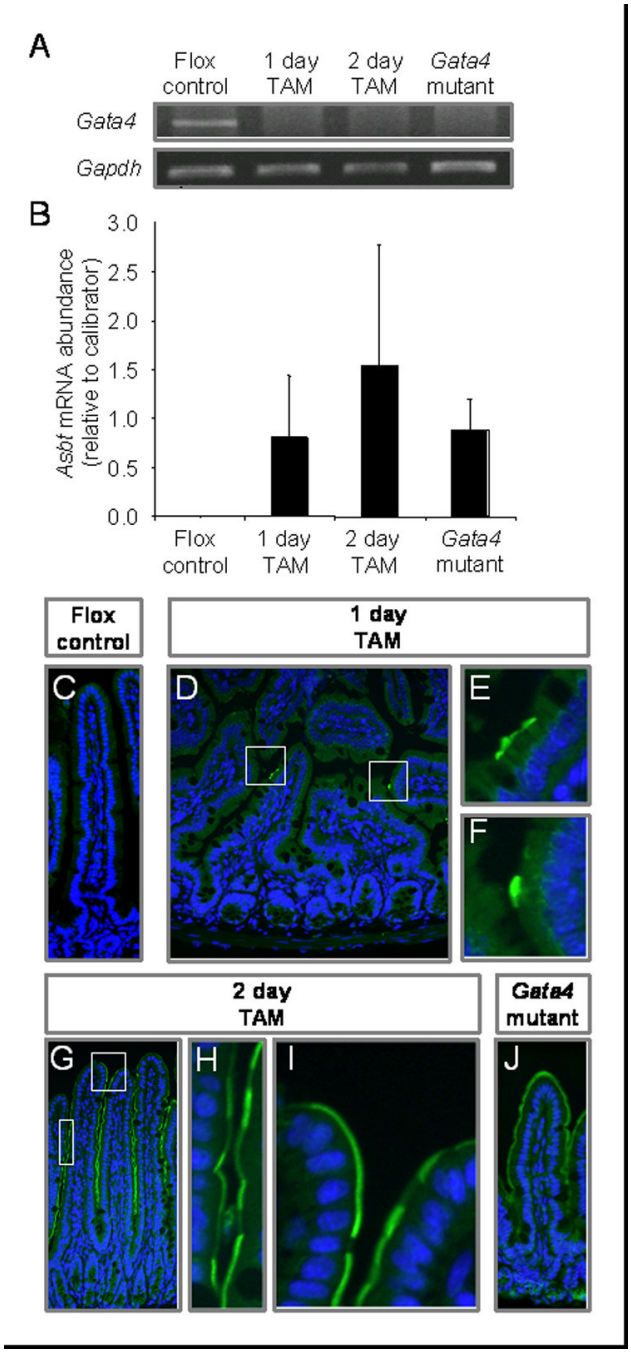

Fig. 2.

GATA4-mediated repression of *Asbt* expression occurs in differentiated absorptive enterocytes. *Gata4*$^{flox/flox}$, Villin*Cre*ER^{T2}-positive mice were treated with tamoxifen for 1 day (1 day TAM) or 2 days (2 day TAM) and sacrificed 24 h after the last injection. *Gata4*$^{flox/flox}$ mice, negative (Flox control) or positive (*Gata4* mutant) for the Villin*Cre*ER^{T2} transgene were treated with tamoxifen for 5 days and sacrificed 2 wk later as described (Bosse et al., 2006a). All samples were collected from the geometric center of the small intestine (mid-jejunum). (A) *Gata4* mRNA abundance, determined by semi-quantitative RT-PCR using primers specific for exon2, reveals complete CRE-mediated excision after only one treatment of tamoxifen. *Gapdh* was used as a positive control. This finding was

replicated on 3 different sets of mice. (B) *Asbt* mRNA is induced within 1 day of a single dose of tamoxifen as determined by real-time RT-PCR (n=3 in each group). RNA from jejunum of a *Gata4* mutant mouse was used as a calibrator. (C–J) ASBT is induced in the microvillus membrane of enterocytes on villi 1 and 2 days after tamoxifen treatment as determined by immunofluorescence (green). Nuclei were counterstained with DAPI (blue).

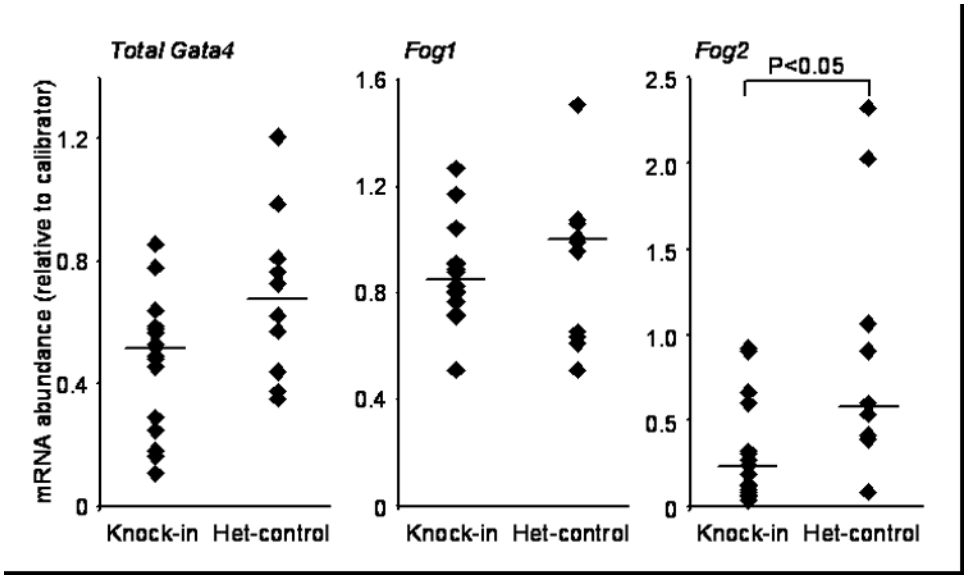

Fig. 3.

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

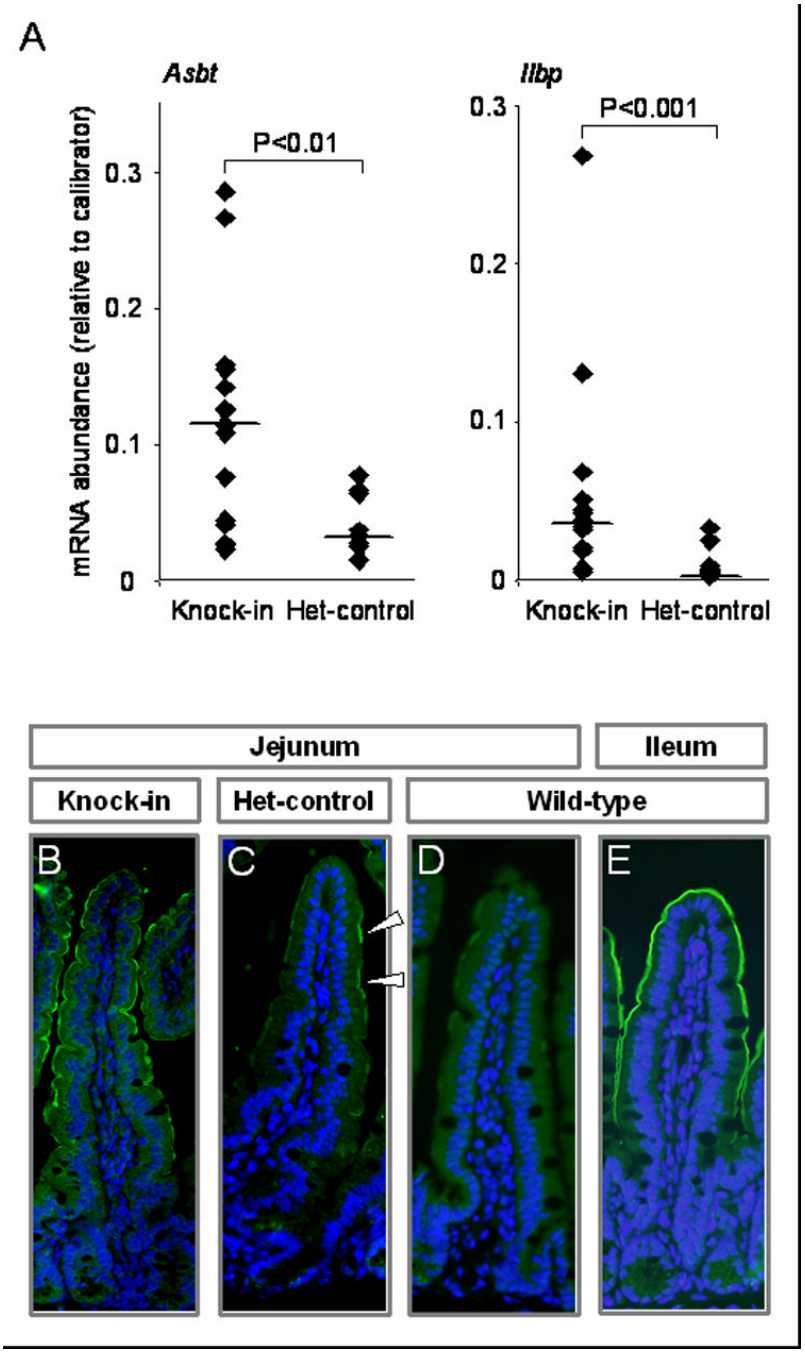

Fig. 4.

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

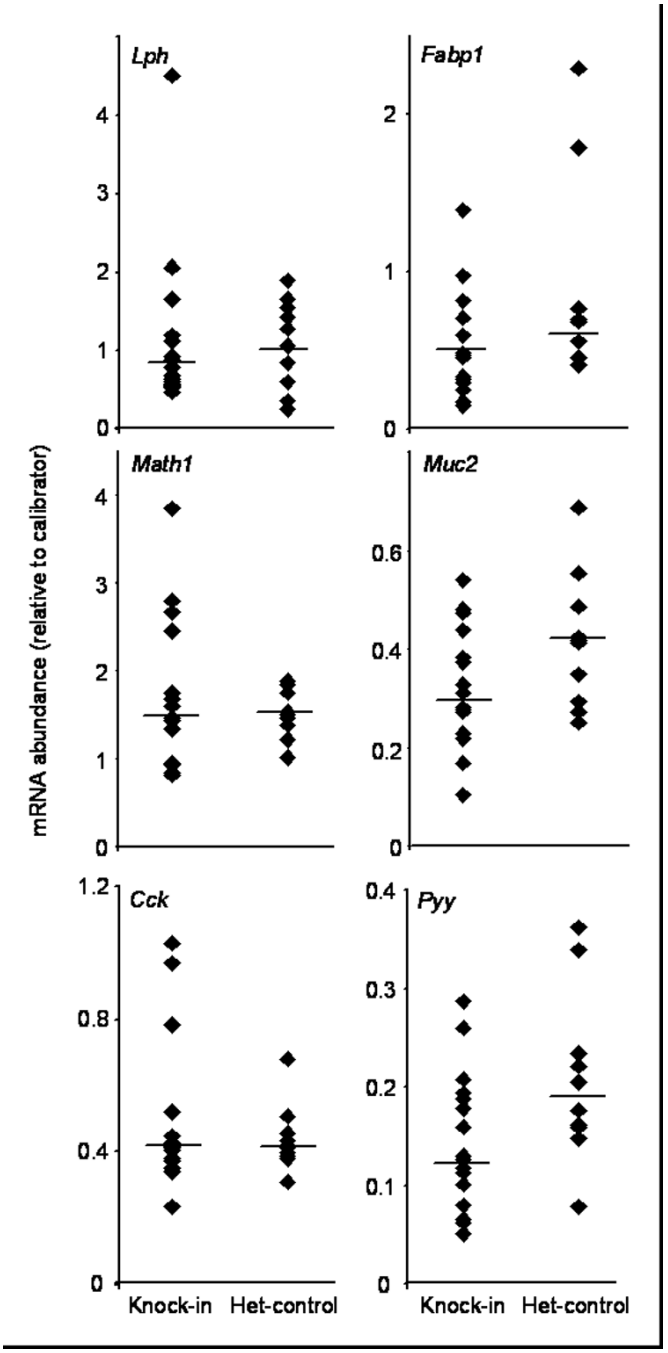

Fig. 5.

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

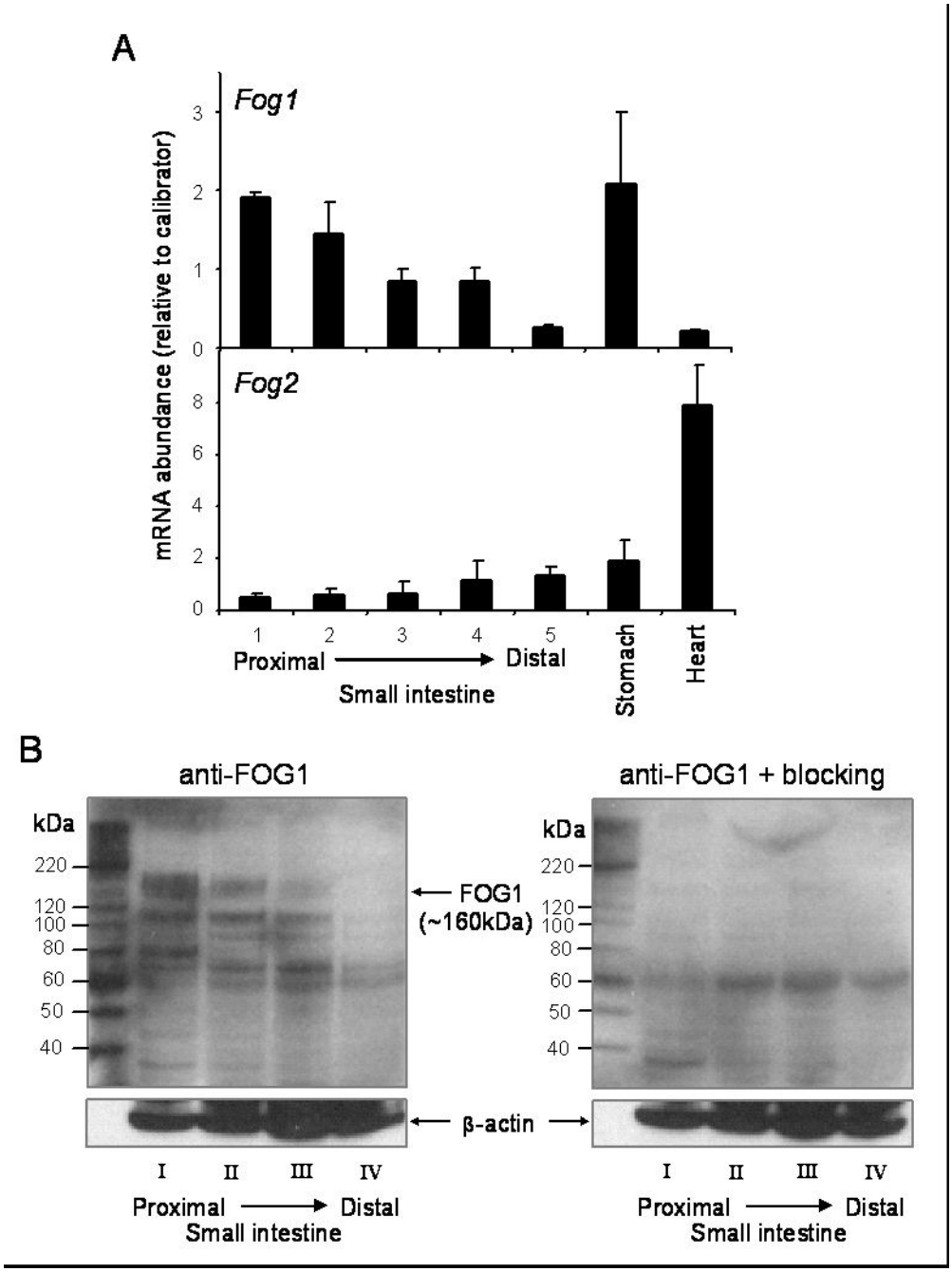

Fig. 6.

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

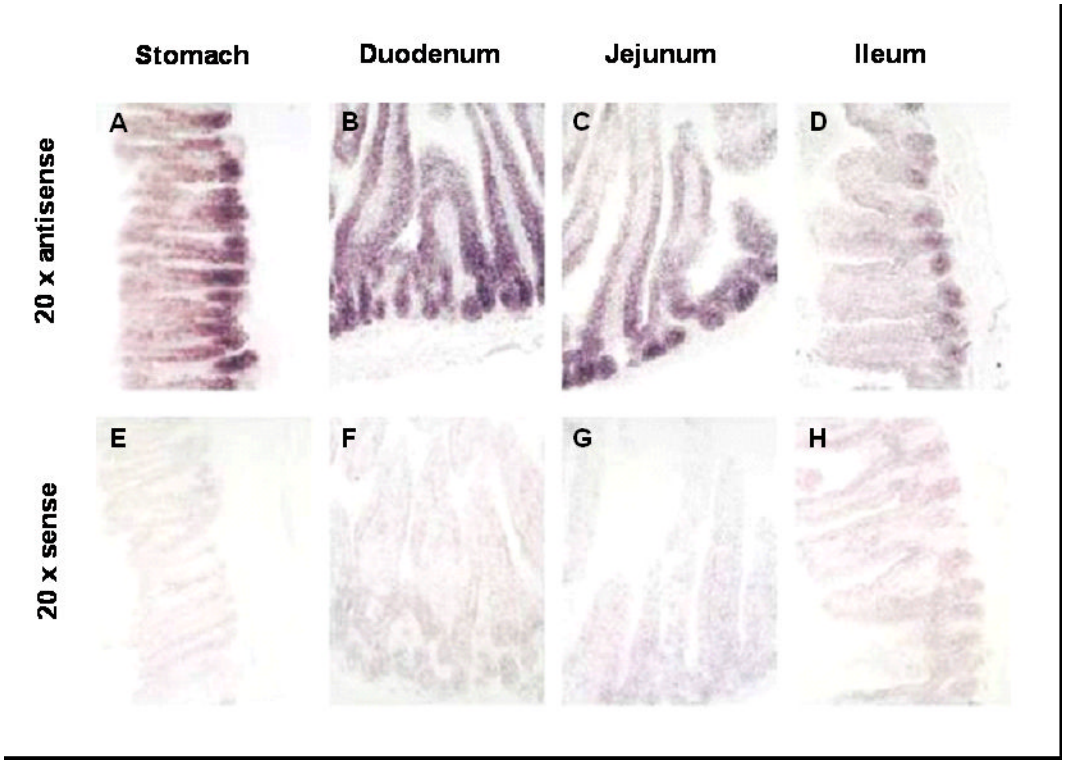

Fig. 7.

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

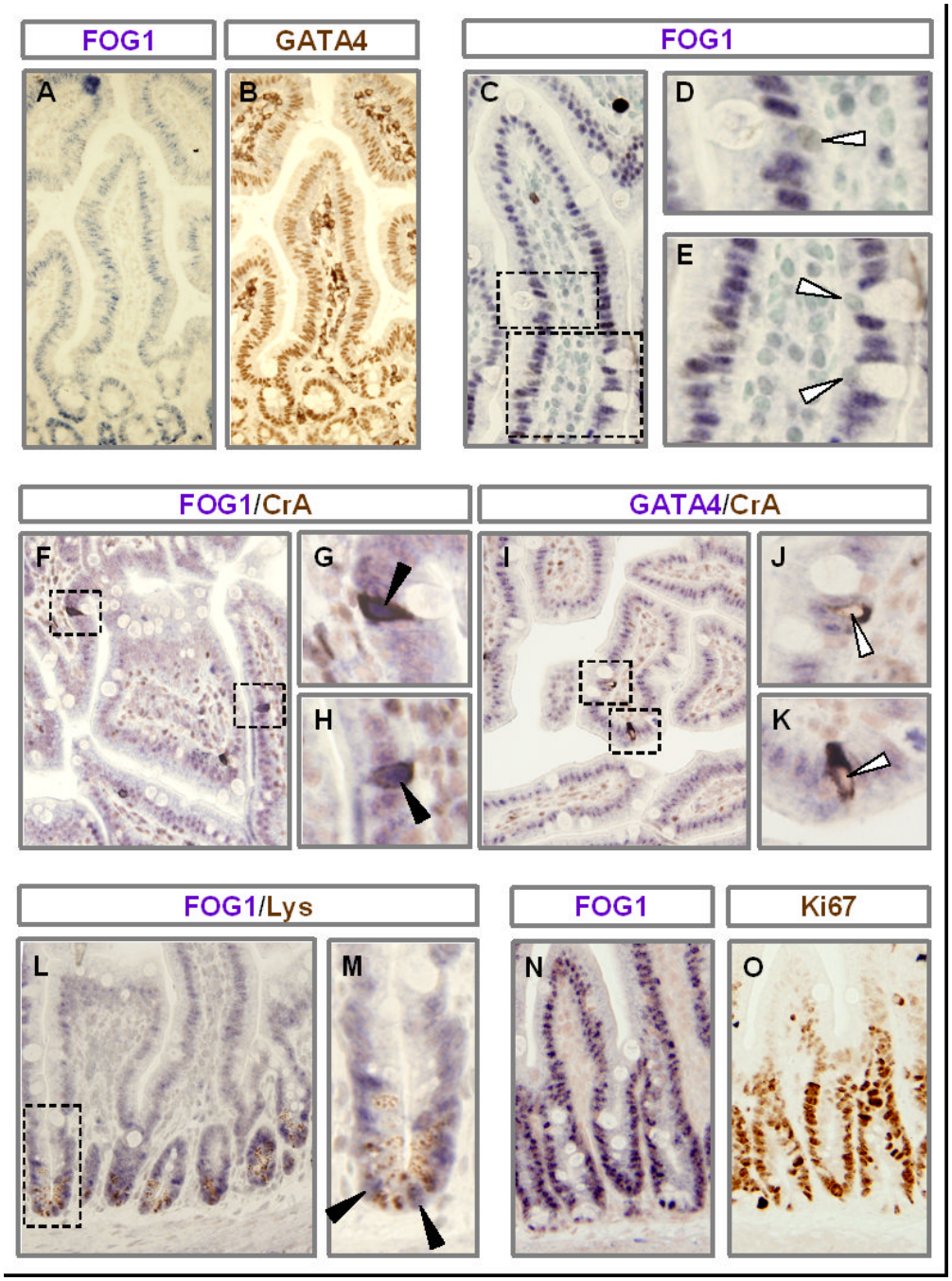

Fig. 8.

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

show that FOG1 is expressed in Ki67-positive proliferating cells in crypts. Open arrowheads indicate an absence of FOG1 or GATA4 staining; filled arrowheads indicate FOG1-positive immunostaining.