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Intestinotrophic Glucagon-Like Peptide-2 (GLP-2) Activates Intestinal Gene Expression and Growth Factor-Dependent Pathways Independent of the Vasoactive Intestinal Peptide Gene in Mice

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The enteroendocrine and enteric nervous systems convey signals through an overlapping network of regulatory peptides that act either as circulating hormones or as localized neurotransmitters within the gastrointestinal tract. Because recent studies invoke an important role for vasoactive intestinal peptide (VIP) as a downstream mediator of glucagon-like peptide-2 (GLP-2) action in the gut, we examined the importance of the VIP-GLP-2 interaction through analysis of $Vip^{-/-}$ mice. Unexpectedly, we detected abnormal villous architecture, expansion of the crypt compartment, increased crypt cell proliferation, enhanced *Igf1* and *Kgf* gene expression, and reduced expression of Paneth cell products in the $Vip^{-/-}$ small bowel. These abnormalities were not reproduced by antagonizing VIP action in wild-type mice, and VIP administration did not reverse the intestinal phenotype of $Vip^{-/-}$ mice. Exogenous administration of GLP-2 induced the expression of ErbB ligands and immediate-early genes to similar levels in $Vip^{+/+}$ *vs.* $Vip^{-/-}$ mice. Moreover, GLP-2 significantly increased crypt cell proliferation and small bowel growth to comparable levels in $Vip^{+/+}$ *vs.* $Vip^{-/-}$ mice. Unexpectedly, exogenous GLP-2 administration had no therapeutic effect in mice with dextran sulfate-induced colitis; the severity of colonic injury and weight loss was modestly reduced in female but not male $Vip^{-/-}$ mice. Taken together, these findings extend our understanding of the complex intestinal phenotype arising from loss of the *Vip* gene. Furthermore, although VIP action may be important for the antiinflammatory actions of GLP-2, the *Vip* gene is not required for induction of a gene expression program linked to small bowel growth after enhancement of GLP-2 receptor signaling. **(*Endocrinology* 153: 0000–0000, 2012)**

Enteroendocrine cells are distributed throughout the stomach and small and large intestine and constitute an important cellular network coordinating the control of nutrient ingestion, gall bladder emptying and pancreatic exocrine secretion, gut motility, energy absorption, and nutrient disposal. These actions are accomplished by the regulated synthesis and secretion of dozens of regulatory peptides that act in a paracrine, neural, and endocrine manner to control energy intake and assimilation. Accordingly, there is considerable interest in understanding how the enteroendocrine network originates signals that communicate with local and distant targets.

Proglucagon-derived peptides (PGDP) represent a subset of enteroendocrine-derived hormones that have attracted considerable interest due to their actions on control of food intake, gastrointestinal motility, and insulin and glucagon secretion (1). Tissue-specific posttranslational processing of proglucagon in pancreas, intestine, and brain underlies the complexity of PGDP production in mammals (2). In the pancreas, the major bioactive PGDP is glucagon, whereas enteroendocrine cells produce oxyn-

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Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; DS, dextran sulfate; GLP, glucagon-like peptide; GLP-2R, GLP-2 receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; PGDP, proglucagon-derived peptide; TNBS, 2,4,6-trinitrobenzene sulfonic acid; VIP, vasoactive intestinal peptide; VPAC1, VIP receptor type 1; WT, wild type.

tomodulin, glicentin, and two glucagon-like peptides (GLP), GLP-1 and GLP-2 (3). Within the gastrointestinal tract, GLP-1 engages the enteric nervous system, leading to control of gut motility and activation of a gut-brain axis that regulates blood flow, insulin secretion, and glucose disposal in peripheral tissues (4). The diverse glucoregulatory actions of GLP-1 underlie the development of medications based on potentiation of GLP-1 action for the treatment of type 2 diabetes (5).

GLP-2 cosecreted together with GLP-1 from L cells acts more proximally in the gut to enhance nutrient absorption (6). Exogenous GLP-2 administration also expands the mucosal surface area of the small bowel by stimulating crypt cell proliferation and inhibiting enterocyte apoptosis (7). GLP-2 also engages antiinflammatory pathways in the intestinal mucosa, and administration of GLP-2 receptor (GLP-2R) agonists attenuates intestinal inflammation in multiple preclinical models of gut injury (8–15). The actions of GLP-2 to promote nutrient absorption, reduce gut motility, and decrease intestinal injury have prompted assessment of whether GLP-2R agonists might be useful for the treatment of human subjects with short bowel syndrome or inflammatory bowel disease (16, 17).

Despite a considerable number of studies describing actions of GLP-2 on crypt cells, immune cells, and enterocytes, the precise mechanisms mediating the actions of GLP-2 within the gut mucosa remain incompletely understood (18). A single receptor transducing the actions of GLP-2 has been identified that exhibits considerable amino acid and structural identity with related members of the class B G protein-coupled receptor family (19). Localization of the GLP-2R to specific cell types in the gut has been challenging, in part due to the low level of GLP-2R expression, and the quality of antisera required for detection of the GLP-2R with high sensitivity and specificity. The GLP-2R has been localized to enteric neurons, myofibroblasts, and subsets of enteroendocrine cells in studies of the rodent, porcine, and human gastrointestinal tract (18). Surprisingly, however, GLP-2Rs have not been localized to crypt cells or enterocytes, implying that many of the effects of GLP-2 are indirect, generated by one or more downstream effectors liberated from GLP-2R$^+$ cell types.

Analyses of mechanisms mediating GLP-2 action in the small and large intestine have employed receptor antagonists, immunoneutralizing antisera, and knockout mice. To date, keratinocyte growth factor, IGF-I, and members of the ErbB superfamily have been implicated as growth factors transducing the proliferative effects of GLP-2 in the gut (20–22). In contrast, the mechanisms through which GLP-2 exerts its antiinflammatory actions are less well understood. However, several studies have identified vasoactive intestinal peptide (VIP) as a GLP-2-activated target that contributes to amelioration of inflammation after GLP-2 administration in the 2,4,6-trinitrobenzene sulfonic acid (TNBS) or dextran sulfate (DS) models of rat colitis (11).

VIP is a 28 amino acid peptide that is widely expressed in the central, peripheral, and enteric nervous systems. Within the gut, VIP regulates gut motility, and disturbances of VIP innervation have been implicated in the pathophysiology of irritable bowel syndrome. VIP also exhibits cytoprotective and vasoactive actions and displays immunomodulatory activity in experimental models of inflammation, predominantly in the central and peripheral nervous system. Many of the actions ascribed to VIP overlap those identified for pituitary adenylate cyclase-activating polypeptide (PACAP), a 28-amino-acid neuropeptide that exhibits considerable amino acid identity with VIP. Furthermore, both VIP and PACAP are capable of activating three structurally related receptors, albeit with varying potency, providing considerable complexity in attribution of precise biological actions to a single peptide and receptor (23–25). Because VIP and GLP-2 exhibit an overlapping spectrum of actions that encompass effects on blood flow, inflammation, and cell proliferation and survival, and VIP has been implicated as a downstream target of GLP-2 action (11, 26), we have now examined the requirement for VIP in a spectrum of GLP-2 actions in $Vip^{-/-}$ mice.

Materials and Methods

Animals

C57BL/6 VIP/peptide histidine isoleucine knockout mice ($Vip^{-/-}$) have been described previously (27). $Vip^{+/+}$ littermates were used as controls for all studies involving $Vip^{-/-}$ mice. Except where indicated, studies were performed on female mice aged 8–12 weeks bred at the Toronto Centre for Phenogenomics animal facility. Wild-type (WT) mice of the C57BL/6 background were obtained from the in house colony at the Toronto Centre for Phenogenomics. C57BL/6J-$Apc^{Min/+}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and crossed with $Vip^{-/-}$ mice to generate $Apc^{Min/+}$:$Vip^{+/-}$ mice, which were then bred to generate $Apc^{Min/+}$/$Vip^{+/+}$ and $Apc^{Min/+}$/$Vip^{-/-}$ mice. Polyp burden was assessed in littermate female mice at 14–15 wk of age. All animal experiments were approved by the Animal Care Committee of the Mount Sinai Hospital.

Peptide and drug treatments

Human [Gly^2] GLP-2, hence referred to as GLP-2, was from Pepceutical Ltd. (Nottingham, UK), VIP was purchased from Bachem (Torrance, CA) and the VIP receptor antagonist [Lys^1-Pro^{2,5}-Arg^{3,4}-Tyr^6] VIP (VIP hybrid) (28) from Sigma-Aldrich (Oakville, Ontario, Canada). Peptides were dissolved in PBS (vehicle) and administered to mice by sc injection. Experiments involving analysis of DS-induced colitis were carried out as pre-

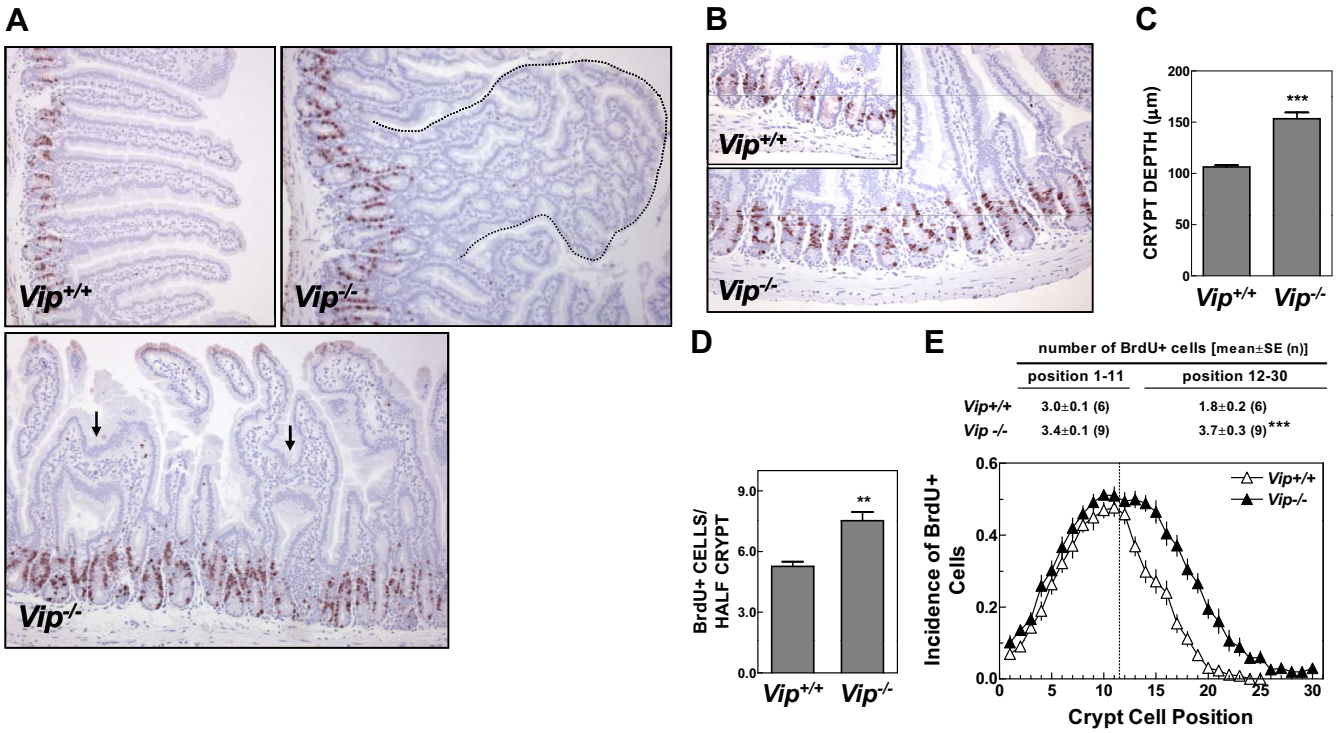

FIG. 1. Abnormal villus-to-crypt axis architecture in the jejunum of the *Vip*$^{-/-}$ mouse. A and B, Photomicrographs illustrating typical villous aberrations (A), including intervillous bridges (*arrows*) and a spongiform polyp (*outlined with a dotted line*), and the distinct enlarged crypt compartment (B) in the jejunum of the *Vip*$^{-/-}$ mouse. Images were obtained from tissue sections stained for BrdU and counterstained with hematoxylin. C–E, Crypt depth (C) and crypt cell proliferation (D and E) in the jejunum of *Vip*$^{-/-}$ mice and WT littermate controls. Tissue sections were scored for total number of BrdU^{+} cells per half-crypt (D) and incidence of BrdU staining at each cell position within the crypt (E). Position 1 corresponds to the base of the crypt. For C–E, n = 6–9 mice per group combined from two independent experiments. **, $P < 0.0$; ***, $P < 0.001$, *Vip*$^{-/-}$ *vs. Vip*$^{+/+}$.

viously described (15). A clinical disease activity index ranging from 0–4 (29) was determined by scoring stool consistency, presence or absence of fecal blood, and weight loss. To assess intestinal crypt cell proliferation, 5-bromo-2′-deoxyuridine (BrdU) (Sigma-Aldrich; 100 mg/kg) dissolved in PBS was injected ip to mice 1 h before death.

Tissue collection, morphometry, immunohistochemistry, and polyp evaluation

Small intestine and colon were collected, flushed with PBS to remove luminal contents, and weighed, and lengths were measured under constant tension. Adjacent 2-cm intestinal segments were obtained from jejunum and colon and fixed in 10% neutral-buffered formalin and paraffin embedded or snap-frozen in liquid nitrogen and stored at −70 C. Digital image acquisition and morphometry were done on 5-μm histological sections stained with hematoxylin and eosin as described (22, 30, 31). Immunohistochemistry was carried out using indirect immunoperoxidase detection with NovaRED substrate (Vector Laboratories, Burlington, Ontario, Canada) followed by hematoxylin counterstaining. A rabbit polyclonal antilysozyme antibody (DakoCytomation, Mississauga, Ontario, Canada) was used to detect Paneth cells. BrdU immunopositivity was detected using a mouse monoclonal anti-BrdU antibody (Invitrogen Canada, Burlington, Ontario, Canada). The incidence of BrdU staining at each cell position within the crypt was scored in a minimum of 100 half-crypts per mouse. To assess polyp burden in *Apc*$^{Min/+}$ mice, the small and large intestines were removed and flushed with PBS. The small intestine was divided into three equal segments (proximal, middle, and distal). Then, the intestines were opened longitudinally, laid flat on Whatman paper, and fixed in 10% neutral-buffered formalin for 24 h. Fixed intestines were stained with methylene blue and examined for polyps with the use of a dissection microscope equipped with an eyepiece micrometer.

Western blot analysis

Whole-tissue extracts were prepared by homogenization of intestinal segments in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate in PBS) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich), 5 mM sodium fluoride, and 200 μM sodium orthovanadate. Protein (30–45 μg) was used for Western blot analysis as described (22, 31). The rabbit polyclonal antibody reactive to lysozyme was from DakoCytomation. A mouse monoclonal antibody against heat-shock protein 90 (BD Biosciences, Mississauga, Ontario, Canada) was used as a loading control.

RNA isolation and quantitative real-time RT-PCR

Total RNA from intestinal tissue was extracted by the guanidinium thiocyanate method and cDNA synthesis performed with random hexamers and SuperScript II (Invitrogen Canada). Real-time quantitative PCR was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems) for am-

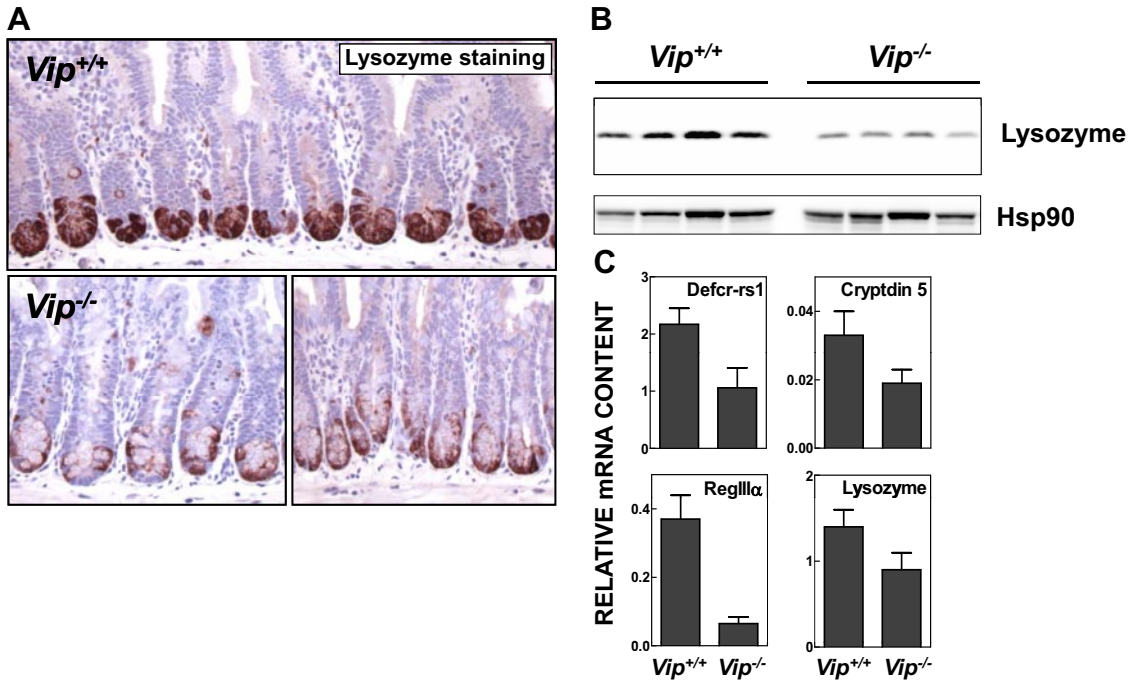

FIG. 2. Reduced expression of Paneth cell-specific products in the jejunum of the $Vip^{-/-}$ mouse. A, Immunohistochemical detection of lysozyme in the jejunal crypt compartment of mice of the indicated *Vip* genotype. Photomicrographs are representative of four to six mice per group. B, Western blot analysis of lysozyme expression in whole-tissue jejunal extracts from four $Vip^{-/-}$ mice and four WT littermates. Anti-heat-shock protein 90 (Hsp90) antibody was used to monitor loading and transfer conditions. C, Relative mRNA levels of Paneth cell markers in the jejunum of $Vip^{-/-}$ and $Vip^{+/+}$ mice as assessed by real-time quantitative RT-PCR (n = 8–12 mice per group combined from three independent experiments).

phiregulin (Mm00437583_m1), cryptdin 5 (Mm00651548_g1), *defcr-rs1* (Mm00655850_m1), *egf* (Mm00438696_m1), *egr-1* (Mm00656724_m1), epiregulin (Mm00514794_m1), c-*fos* (Mm00487425_m1), *glp2r* (Mm01329473_m1), *hb-egf* (Mm00439307_m1), *igf-1* (Mm00439560_m1), *kgf* (Mm00433291_m1), lysozyme P (Mm00657323_m1), *pacap* (Mm00437433_m1), *phlda-1*, (Mm00456345_g1), proglucagon (Mm00801712_m1), *regIIIα* (Mm00441121_m1), *tff3* (Mm00495590_m1), and *tgfa* (Mm00446232_m1). Relative quantification of transcript levels was performed by the $2^{-\Delta Ct}$ method using the cycle threshold (Ct) values obtained from the PCR amplification kinetics with the ABI PRISM SDS version 2.1 software. The 18S rRNA was used for normalization because its intestinal expression remained unaltered regardless of mouse genotype or treatment.

Statistics

Results are expressed as mean ± SE. Statistical significance was assessed by ANOVA followed by the Bonferroni *post hoc* test and, where appropriate, by unpaired Student's *t* test using GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

Results

Because previous experiments assessing the importance of VIP as a downstream mediator of GLP-2 action have employed peptide antagonists that may incompletely attenuate VIP action (11, 32), we initiated studies using $Vip^{-/-}$ mice (27). We first examined the baseline phenotype of the $Vip^{-/-}$ gut. Unexpectedly, $Vip^{-/-}$ jejunum exhibited aberrant villous architecture, with a high incidence of spongiform polyps and intervillous bridges, consistent with villous fusion, a rare histological abnormality occasionally observed in the setting of enteritis (Fig. 1A). Crypt cell proliferation within the murine small bowel is classically quantified by assessing the position and number of proliferating cells expressing endogenous markers of cell cycle progression (Ki67, or proliferating cell nuclear antigen) or by determination of the number of cells that have taken up BrdU (33). Both crypt depth (Fig. 1, B and C) and crypt cell proliferation (Fig. 1D) were significantly increased in the jejunum of $Vip^{-/-}$ mice. Analysis of the number and distribution of proliferating cells within the crypt compartment revealed a significant shift in the number and position of $BrdU^{+}$ cells in $Vip^{-/-}$ jejunum (Fig. 1E).

The disordered control of small bowel growth in $Vip^{-/-}$ mice prompted us to examine the mRNA levels of key peptides and growth factors in the small *vs.* the large bowel of $Vip^{-/-}$ *vs.* $Vip^{+/+}$ mice. Levels of *PACAP*, *Igf1*, and *KGF* mRNA transcripts were significantly up-regulated, whereas levels of proglucagon were reduced in the $Vip^{-/-}$ small bowel (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). In contrast, both proglucagon (*Gcg*), and *Glp2r* mRNA transcripts were increased in the

Vip$^{-/-}$ colon, whereas no changes in levels of *TGFα*, *EGF*, or *Tff3* mRNA transcripts were detected in the small or large bowel of *Vip*$^{-/-}$ mice.

A reduced intensity of lysozyme-positive Paneth cells was observed by immunohistochemical analysis at the crypt base in the *Vip*$^{-/-}$ small bowel (Fig. 2A). Consistent with these findings, levels of lysozyme were clearly reduced in jejunal extracts from *Vip*$^{-/-}$ compared with *Vip*$^{+/+}$ mice (Fig. 2B). We recently described reduced expression of antimicrobial gene products and reduced bactericidal activity in mucosal extracts of *Glp2r*$^{-/-}$ mice, findings consistent with a defect of Paneth cell activity (34). Because Paneth cells play an important specialized role in the defense against microbial-induced intestinal injury within the gastrointestinal tract (35), we assessed the expression of mRNA transcripts for key Paneth cell genes in the *Vip*$^{-/-}$ small bowel. Analysis of Paneth cell gene products revealed reduced levels of not only lysozyme P but also of *Defcr-rs1*, cryptdin 5, and *RegIIIα* mRNA transcripts in RNA from *Vip*$^{-/-}$ jejunum (Fig. 2C). Taken together, these findings extend previous descriptions of the basal intestinal phenotype of *Vip*$^{-/-}$ mice (36).

The increased rates of crypt cell proliferation in *Vip*$^{-/-}$ jejunum may reflect ongoing VIP deficiency and/or developmental changes arising from embryonic absence of *Vip* gene products during mouse development. To determine the reversibility of this phenotype, we treated mice with exogenous VIP and reassessed crypt cell proliferation. VIP administration had no effect on crypt depth or crypt cell proliferation in *Vip*$^{-/-}$ mice (Fig. 3A). To ascertain whether diminution of VIP action in WT mice would recapitulate the intestinal findings observed in *Vip*$^{-/-}$ mice, we treated WT mice with a VIP peptide hybrid antagonist, 100 μg/kg, for 25 d (11). Administration of the VIP hybrid alone for 25 d had no effect on crypt cell proliferation in WT mice (Fig. 3B). Furthermore, the ability of GLP-2 to robustly increase jejunal crypt cell proliferation was unaltered in mice chronically treated with the antagonist VIP hybrid (Fig. 3B). Hence, reduction of VIP action does not affect basal or GLP-2-stimulated control of crypt cell proliferation. To ascertain the potential biological significance of the increased rate of crypt cell proliferation observed in *Vip*$^{-/-}$ mice in a sensitized model of tumor formation, we generated *Apc*$^{Min/+}$:*Vip*$^{-/-}$ mice and assessed the polyp burden within the intestines. No significant differences in polyp number or size were observed in *Apc*$^{Min/+}$:*Vip*$^{-/-}$ *vs.* *Apc*$^{Min/+}$:*Vip*$^{+/+}$ mouse intestines (Supplemental Fig. 2).

Because GLP-2 and VIP exert antiinflammatory, proliferative, and cytoprotective actions in the gastrointestinal tract, we further assessed the requirement for VIP as a downstream mediator of GLP-2 action in the murine gut.

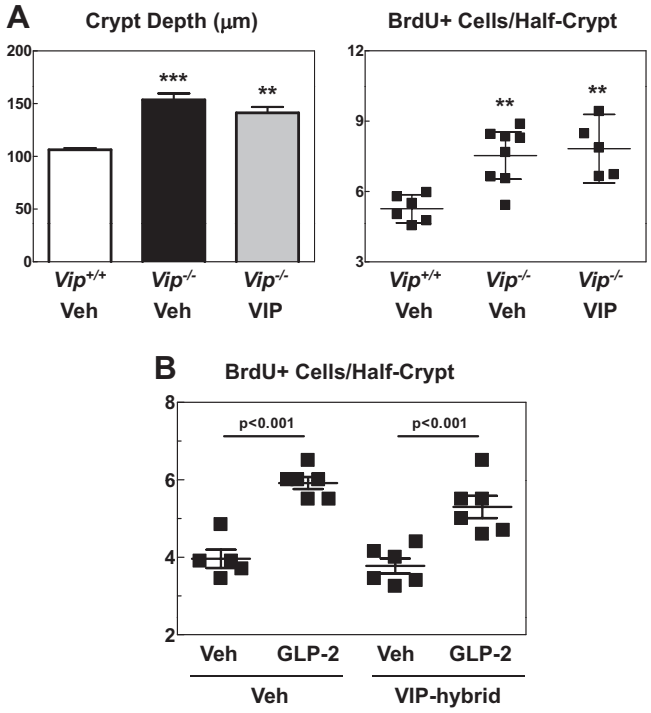

FIG. 3. A, Jejunal crypt depth and crypt cell proliferation after the administration of VIP (2 nmol per mouse) or vehicle alone (Veh) every other day for 11 d in *Vip*$^{-/-}$ and *Vip*$^{+/+}$ littermates as indicated (n = 5–8 mice per group). Each data point in the *right panel* corresponds to one mouse. **, $P < 0.01$; ***, $P < 0.001$, *Vip*$^{-/-}$, either VIP- or vehicle-treated, *vs.* *Vip*$^{+/+}$. B, Basal (vehicle-treated) *vs.* GLP-2-stimulated jejunal crypt cell proliferation in WT C57BL6 mice administered VIP hybrid (100 μg/kg once a day) or vehicle alone (Veh) for 25 d. BrdU labeling was assessed after treatment for 6 h with GLP-2 (0.2 mg/kg, injected every 3 h) or vehicle. Each data point corresponds to one mouse (n = 5–6 mice per group). The statistical significance for the comparison GLP-2 *vs.* vehicle is indicated.

Acute GLP-2 administration robustly increased the abundance of mRNA transcripts for immediate-early genes and ErbB ligands, including *egr-1*, *c-fos*, *Phlda-1*, amphiregulin, epiregulin, and *Hb-EGF* to a comparable level in *Vip*$^{+/+}$ *vs.* *Vip*$^{-/-}$ mice (Fig. 4A). Consistent with previous findings, acute GLP-2 administration significantly increased crypt cell proliferation in the jejunum of *Vip*$^{+/+}$ mice (Fig. 4B). Although the basal rate of crypt cell proliferation was higher in *Vip*$^{-/-}$ *vs.* *Vip*$^{+/+}$ mice, GLP-2 significantly increased crypt cell proliferation in *Vip*$^{-/-}$ mice (Fig. 4, B and C). Furthermore, administration of GLP-2 (0.2 mg/kg) once daily for 9 d produced significant increases in small bowel weight and jejunal villous height in both *Vip*$^{+/+}$ and *Vip*$^{-/-}$ mice (Fig. 5). Jejunal crypt depth and colon weight trended higher but were not significantly different in GLP-2-treated mice (Fig. 5, B and D). Hence, VIP is not required for the GLP-2-stimulated induction of ErbB ligands, immediate-early gene expression, crypt cell proliferation, or small bowel growth.

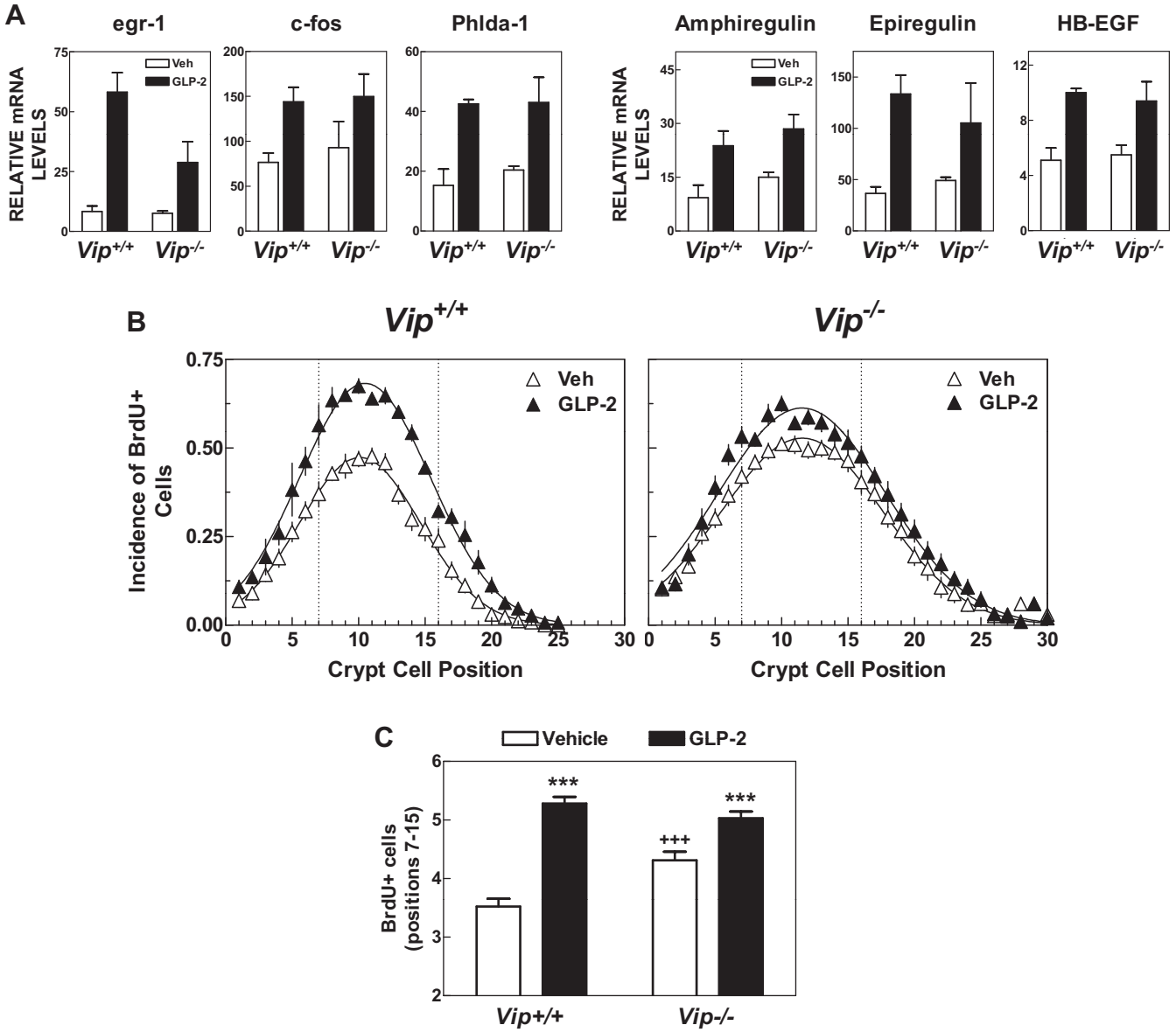

FIG. 4. The acute jejunal response to GLP-2 is preserved in the $Vip^{-/-}$ mouse. A, Levels of mRNA transcripts of immediate-early genes (*egr-1*, c-*fos*, and *Phlda-1*) and ErbB ligands (amphiregulin, epiregulin, and *HB-EGF*) were quantified by real-time quantitative RT-PCR in total RNA from jejunum of $Vip^{-/-}$ mice and WT littermates treated for 45 min with GLP-2 (0.2 mg/kg) or vehicle alone (n = 4 mice per group combined from two independent experiments). B and C, BrdU labeling in the jejunum of $Vip^{-/-}$ mice and $Vip^{+/+}$ littermates after treatment for 6 h with GLP-2 (0.2 mg/kg, injected every 3 h) or vehicle alone (Veh). Incidence of $BrdU^+$ cells along the longitudinal crypt axis (B) and total number of $BrdU^+$ cells scored in positions 7–15 (C). Position 1 corresponds to the base of the crypt. Data are combined from two independent experiments with a total of six to nine mice per group. To facilitate visual comparisons, the crypt BrdU positional data were fitted to a Gaussian function that is indicated by a *solid line*. Two-way ANOVA indicated that the crypt BrdU positional distribution curves from vehicle- and GLP-2-treated mice were significantly different ($P < 0.001$) for both *Vip* genotypes. ***, $P < 0.001$, GLP-2 *vs.* vehicle; +++, $P < 0.001$, $Vip^{-/-}$ *vs.* $Vip^{+/+}$.

Although GLP-2 action is comparatively greater in the small relative to the large intestine, exogenous GLP-2 administration stimulates colonic growth (20) and attenuates injury to the colonic mucosa in mice with DS-induced colitis (10, 15). Furthermore, previous studies have implicated VIP as an essential downstream antiinflammatory mediator of GLP-2 action in rodents with experimental intestinal injury (11). Accordingly, we assessed the severity of intestinal injury in $Vip^{+/+}$ *vs.* $Vip^{-/-}$ mice with DS-induced colitis in the presence or absence of concomitant GLP-2 administration. No difference in weight loss (Fig. 6A) or in the severity of colitis (Fig. 6B) was observed across genotypes in the presence or absence of GLP-2 administration, although untreated female $Vip^{-/-}$ mice exhibited resistance to DS administration. In contrast, GLP-2 treatment stimulated small intestinal growth in mice given DS (Supplemental Fig. 3).

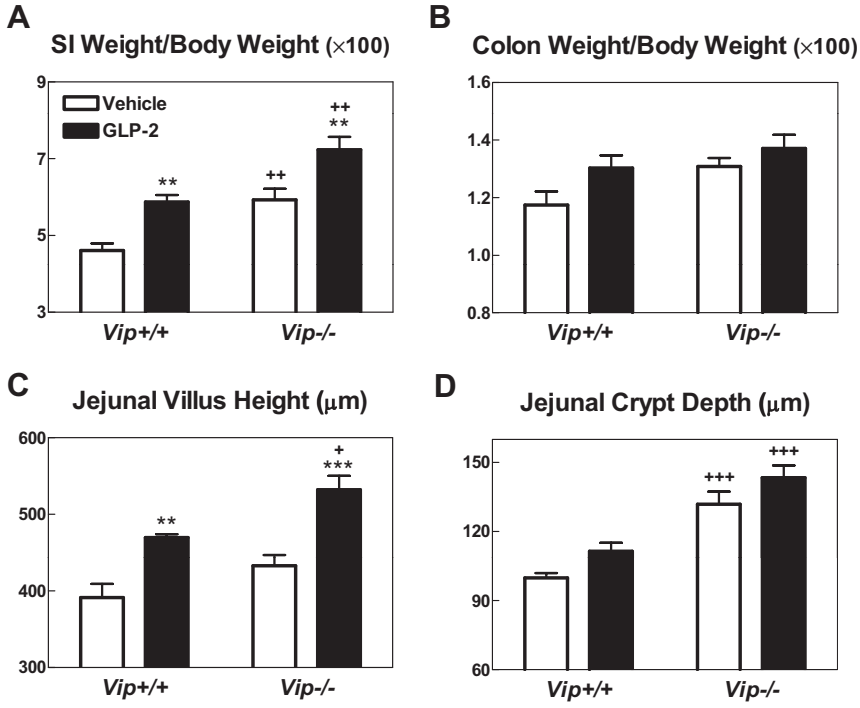

FIG. 5. Relative small intestine (SI) (A) and colonic (B) weight and jejunal histomorphometry (C and D) after the administration of vehicle or GLP-2 (0.2 mg/kg once a day) for 9 d in $Vip^{+/+}$ and $Vip^{-/-}$ littermates (n = 7 mice per group combined from two independent experiments). **, $P < 0.01$; ***, $P < 0.001$, GLP-2 *vs.* vehicle; +, $P < 0.05$, ++, $P < 0.01$; +++, $P < 0.001$, $Vip^{-/-}$ *vs.* $Vip^{+/+}$. No differences in the small intestine length were found between genotypes or treatments.

Discussion

The $Vip^{-/-}$ mouse exhibits a number of interesting phenotypes, including disturbances of circadian rhythm (27), impaired or enhanced inflammatory responses (37, 38), and dysglycemia with abnormal sweet taste preference (39). Very little is known about the basal intestinal phenotype of $Vip^{-/-}$ mice; however, increased small bowel weight and smooth muscle thickening, together with increased villous length and reduced staining of mucus in the small bowel has been described in the $Vip^{-/-}$ mouse (36). Moreover, older $Vip^{-/-}$ mice appeared susceptible to the development of intestinal obstruction. Genetic elimination of the VIP receptor type 1 (*VPAC1*) gene results in impaired neonatal growth, increased mortality of mice around the time of weaning, and increased mucosal cell proliferation and thickening of the bowel wall, detected in mice analyzed at 8 wk of age (40). In contrast, although *VPAC2* knockout mice exhibit enhanced susceptibility to DS-induced colitis (41), a detailed analysis of the basal phenotype of the $VPAC2^{-/-}$ small bowel has not yet been reported. Similarly, although genetic elimination of the *PACAP* gene results in enhanced sensitivity to both small and large bowel injury (42, 43), a small bowel phenotype arising in uninjured $PACAP^{-/-}$ mice has not yet been described.

We now extend our understanding of the importance of *Vip* gene products for small bowel growth by demonstrating enhanced crypt cell proliferation and reduced levels of Paneth cell-specific products in the $Vip^{-/-}$ small bowel. Furthermore, the increased crypt cell proliferation was not reversible by VIP replacement and was not reproduced in WT mice treated with VIP hybrid, a VIP antagonist. Hence, this phenotype likely evolves at least in part due to abnormalities arising from deficiency of one or more *Vip* gene products during development. Interestingly, we demonstrated up-regulation of *PACAP* gene expression in the jejunum and colon of $Vip^{-/-}$ mice (Supplemental Fig. 1), raising the possibility that the intestinal phenotype resulting from ablation of the *Vip* gene is partially masked by compensation from related ligands that activate the same family of VIP/PACAP receptors. Furthermore, up-regulation of *Igf1* and *KGF* gene expression in the $Vip^{-/-}$ small bowel raises the possibility that one or both of these growth factors contributes to the $Vip^{-/-}$ phenotype of increased crypt cell proliferation.

We were interested to examine the importance of VIP as a downstream mediator of GLP-2 action after reports linking VIP to the antiinflammatory actions of GLP-2. Immunohistochemistry and *in situ* hybridization identified a subset of $GLP\text{-}2R^+$ enteric neurons in the porcine and human jejunum, the majority of which also exhibited VIP or endothelial nitric oxide synthase immunoreactivity (44). Subsequent studies demonstrated a functional role for VIP signaling as a downstream mediator of GLP-2 action in rats with TNBS-induced ileitis (11). Both VIP and GLP-2 independently reduced weight loss and myeloperoxidase activity in the inflamed bowel, and in coadministration experiments, a VIP antagonist blocked the antiinflammatory actions of GLP-2 in TNBS-induced enteritis, as documented by increased myeloperoxidase activity, enhanced tissue IL-1β, and failure to suppress IL-10, compared with the effects of GLP-2-treatment alone. Moreover, acute GLP-2 administration significantly increased nuclear c-Fos immunoreactivity in VIP^+ ileal submucosal neurons. Quantification of neuronal populations demonstrated that GLP-2 treatment increases the number of VIP^+ neurons in the absence of inflammation and prevented the loss of VIP^+ neurons in the context

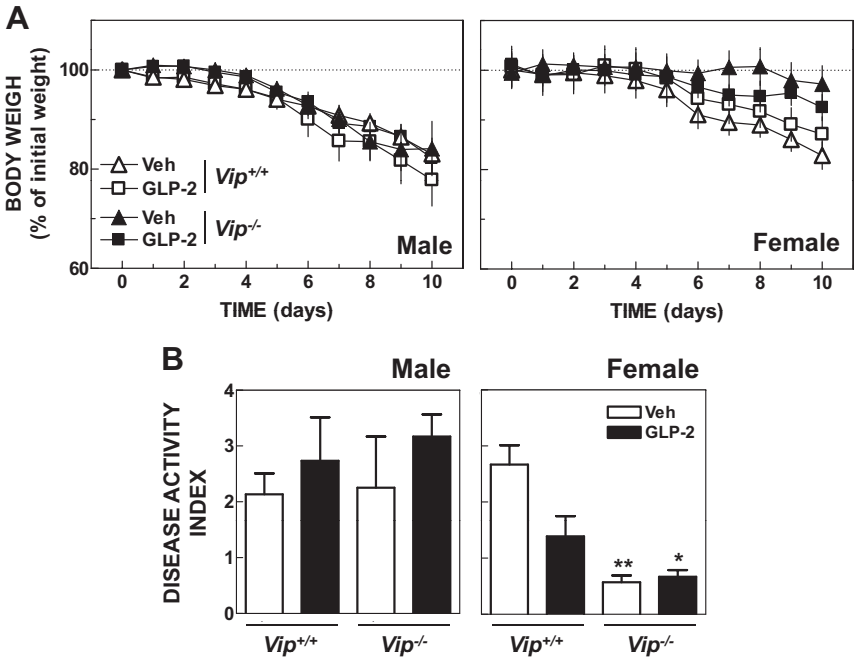

FIG. 6. A, Relative body weight from $Vip^{+/+}$ and $Vip^{-/-}$ male (n = 4–5 per group) and female mice (n = 6–7 per group) given dextran sodium sulfate (2.5% for males and 3.0% for females) in the drinking water for 10 d and administered GLP-2 (0.2 mg/kg) or vehicle once (female) or twice (male) daily. Two-way ANOVA indicated that the weight loss curves from vehicle-treated $Vip^{+/+}$ and $Vip^{-/-}$ female mice were significantly different ($P < 0.001$). B, Disease activity index in $Vip^{+/+}$ and $Vip^{-/-}$ male and female mice after 10 d of oral DS and vehicle or GLP-2 treatment. *, $P < 0.05$; **, $P < 0.01$, GLP-2- and vehicle-treated, respectively, $Vip^{-/-}$ *vs.* vehicle-treated $Vip^{+/+}$.

of active inflammation (26). VIP has also been proposed as a mediator of the gastric myorelaxant activity induced by GLP-2 in the isolated mouse stomach (32).

Previous studies invoking VIP as an essential downstream target for GLP-2 action have employed the VIP receptor antagonist [Lys^1-$Pro^{2,5}$-$Arg^{3,4}$-Tyr^6] VIP (VIP hybrid) (11, 26, 32). However, this molecule is not completely specific for VIP action but binds to multiple VIP/PACAP receptors, predominantly VPAC1 and VPAC2 (45, 46). Hence, this hybrid antagonist molecule blocks not only endogenous VIP but also endogenous PACAP, and one or both peptides may be critical functional mediators of GLP-2 action. We used $Vip^{-/-}$ mice to identify the critical role of *Vip* gene products as downstream targets for GLP-2 action. Our data clearly show that the effects of GLP-2 to induce gene expression, crypt cell proliferation, and small bowel growth do not require the *Vip* gene. Nevertheless, it seems likely that the antiinflammatory actions of GLP-2 described previously are mediated in part by VIP and/or PACAP signaling (11, 26).

Unexpectedly, we did not observe a therapeutic effect of GLP-2 in mice with DS-induced colitis, a model we and others have previously used to characterize the antiinflammatory actions of GLP-2. Notably, previous studies of GLP-2 action in the DS colitis model have used mice in the CD1 background (10, 15), whereas the current study employed mice in the C57BL/6 background. Hence we were unable to assess whether the antiinflammatory actions of GLP-2 in the DS colitis model required VIP due to the lack of GLP-2 efficacy in both male and female $Vip^{+/+}$ and $Vip^{-/-}$ mice with colitis. Furthermore, Sigalet and colleagues (11) did not examine the interaction of GLP-2 and the VIP hybrid antagonist in rats with DS colitis. Hence, the importance of the GLP-2-VIP interaction in this model of colonic inflammation requires further analysis.

In summary, we demonstrate that $Vip^{-/-}$ mice exhibit markedly increased crypt cell proliferation, increased *Igf1* and *KGF* expression, reduced expression of Paneth cell products, and abnormal villous architecture; however, these histological abnormalities are not reversible after VIP replacement. Furthermore, attenuation of VIP signaling with the antagonist VIP hybrid does not reproduce these findings in WT mice, strongly suggesting that the basal phenotype arises secondary to developmental loss of *Vip* gene products. Moreover, although VIP may be an important target for the antiinflammatory actions of GLP-2, the *Vip* gene is not required for the GLP-2-dependent induction of a gene expression program linked to stimulation of crypt cell proliferation and small bowel growth. Additional studies of the relationship linking GLP-2 action to VIP in models of experimental intestinal inflammation may extend our understanding of the importance of VIP as a downstream target for GLP-2 *in vivo*.

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B.Y. and D.H. carried out the experiments, analyzed the data, and wrote the manuscript. B.Y. and D.J.D. designed the experiments, and D.J.D. also wrote the manuscript. J.A.W. generated $VIP^{-/-}$ mice and edited the manuscript.

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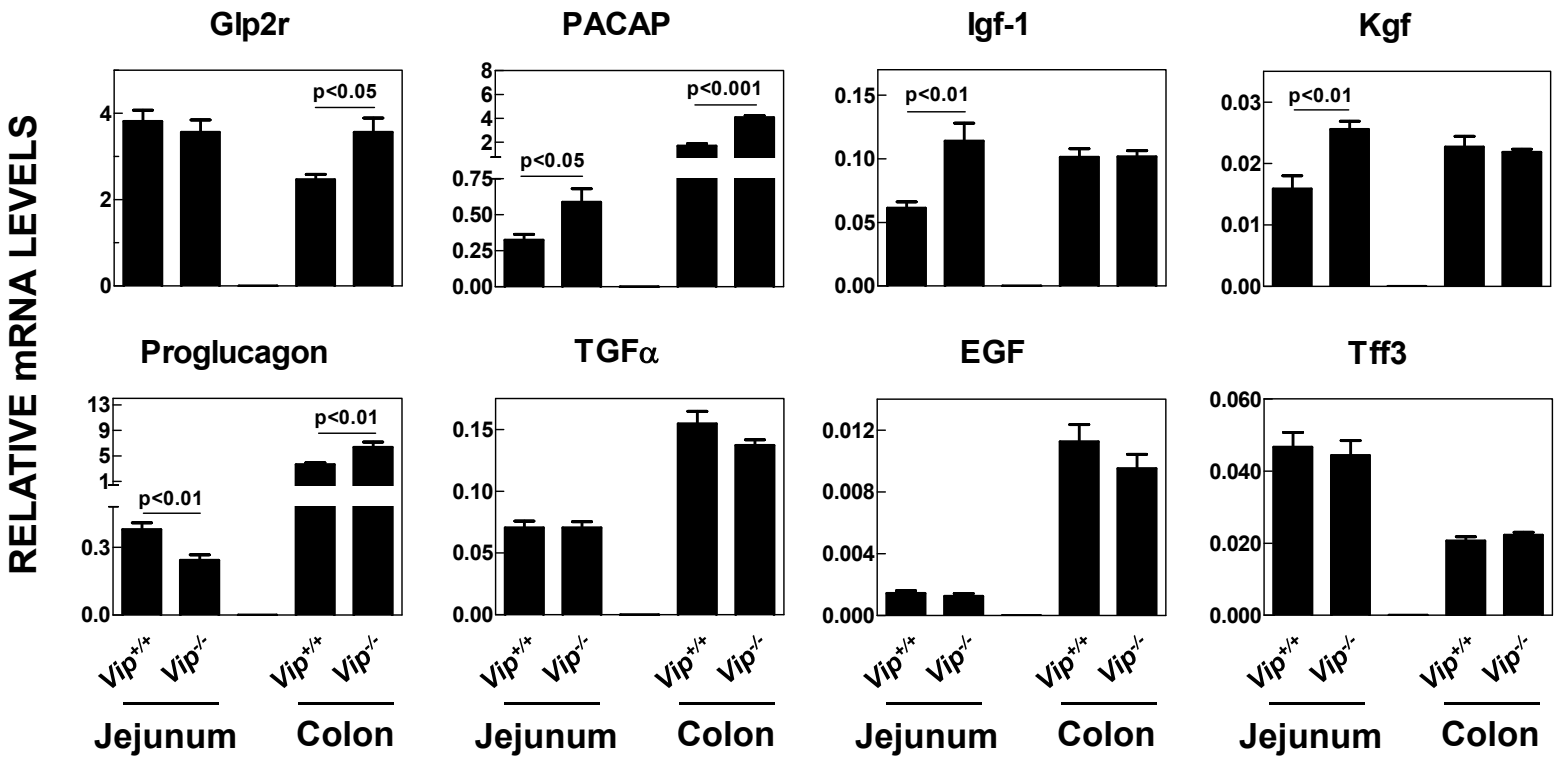
Supplemental Figure Legends

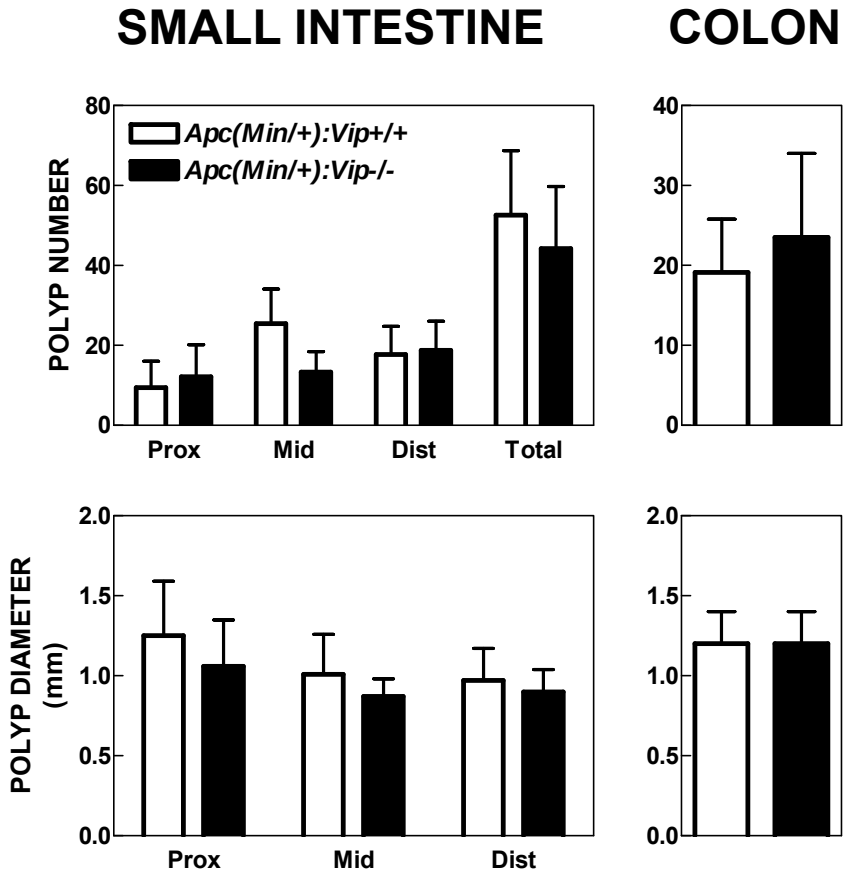
Supplemental Figure 1. Polyp burden in *Apc*$^{Min/+}$ mice is not affected by the *Vip* genotype. Polyp count and average polyp diameter along the gastrointestinal tract in *Apc*$^{Min/+}$:*Vip*+/+ mice (n=12) and *Apc*$^{Min/+}$:*Vip*-/- littermates (n=14). The small intestine was divided into three equal segments (proximal, middle and distal) for polyp assessment.

Supplemental Figure 2. Relative levels of Glp2r and a variety of regulatory peptide/growth factor mRNA transcripts in the jejunum and colon of *Vip*-/- and *Vip*+/+ mice as determined by real-time quantitative RT-PCR (n=6-8 mice per group). The statistical significance for the comparison of *Vip*-/- vs *Vip*+/+ is indicated. Data are representative of at least 3 independent experiments.

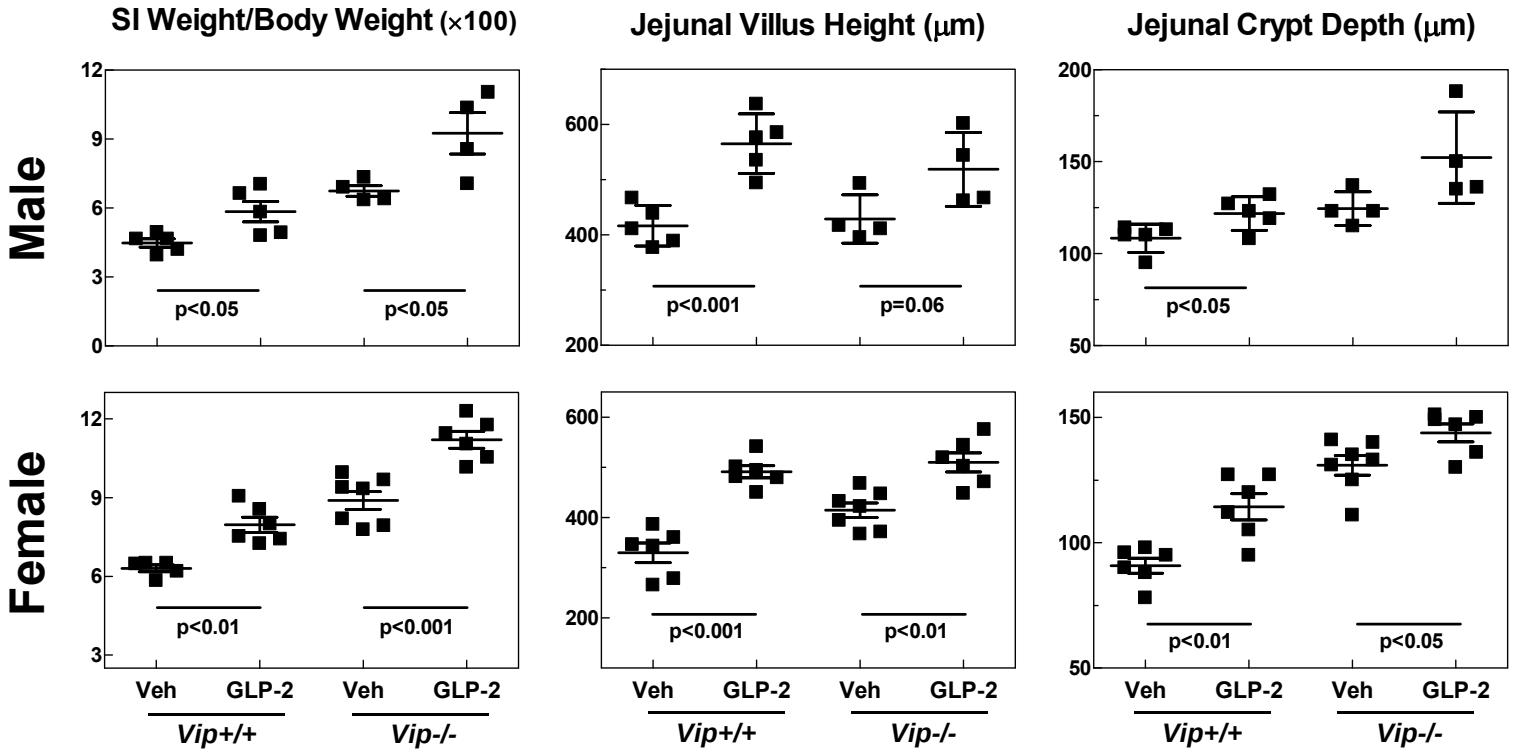
Supplemental Fig 3. Relative small intestine (SI) weight and jejunal villus height and crypt depth in *Vip*+/+ and *Vip*-/- male (top panels) and female (bottom panels) mice after 10 days of oral dextran sulphate and vehicle or GLP-2 treatment as indicated in the legend of Fig 6. Each data point corresponds to one mouse. The statistical significance for the comparisons is shown in each panel.



Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3