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Expression and localization of VPAC1, the major receptor of vasoactive intestinal peptide along the length of the intestine

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Running Title: VPAC1 expression along the length of the intestine

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Abstract

Vasoactive intestinal peptide (VIP) is an endogenous neuropeptide with a broad array of physiological functions in many organs including the intestine. Its actions are mediated via G-protein coupled receptors and VPAC1 is the key receptor responsible for majority of VIP's biological activity. The distribution of VPAC1 along the length of the gastrointestinal tract and its sub cellular localization in intestinal epithelial cells has not been fully characterized. The current studies were undertaken to determine VPAC1 distribution and localization so that VIP based therapies can be targeted to specific regions of the intestine. The results indicated that the mRNA levels of VPAC1 showed an abundance pattern of colon> ileum> jejunum in the mouse intestine. In parallel, the VPAC1 protein levels were higher in the mouse colon, followed by the ileum and jejunum. Immuno-fluorescence studies in mouse colon demonstrated that the receptor was specifically localized to the luminal surface as evident by co-localization with the apical marker villin but not with the basolateral marker Na^+/K^+ ATPase. In the human intestine, VPAC1 mRNA expression exhibited a distribution similar to mouse intestine and was highest in the sigmoid colon. Furthermore, in the human colon, VPAC1 also showed predominantly apical localization. The physiological relevance of the expression and apical localization of VPAC1 remains elusive. We speculate that apical VPAC1 in intestinal epithelial cells may have relevance in recognizing secreted peptides in the intestinal lumen and therefore, supports the feasibility of potential therapeutic and targeting use of VIP formulations via oral route to treat GI diseases.

New and noteworthy

These studies for the first time, present comprehensive data on the relative characterization of VIP receptors in the intestinal mucosa. VPAC1 was identified as the predominant receptor with higher levels in the colon compared to the small intestine and was mainly localized to the apical membrane. In addition, the findings in the human tissues was consistent with VPAC1 expression in the mouse intestine and open possibilities to target colonic tissues with VIP for treating diseases such as IBD.

Keywords: VIP, Mouse intestine, Human intestine, Membrane localization

Abbreviations

VIP- Vasoactive Intestinal Peptide

PACAP- Pituitary Adenylate Cyclase Activating Polypeptide

GIT- Gastrointestinal Tract

KO- Knock Out

VPAC1- Vasoactive Intestinal Peptide Receptor 1

VPAC2- Vasoactive Intestinal Peptide Receptor 2

PAC1- Pituitary Adenylate Cyclase Activating Polypeptide Receptor 1

GPCR- G- Protein Coupled Receptor

IBD- Inflammatory Bowel Disease

cAMP- Cyclic Adenosine Mono Phosphate

NGS- Normal Goat Serum

mRNA- Messenger Ribonucleic Acid

qPCR- Quantitative Polymerase Chain Reaction

GAPDH- Glyceraldehyde 3- Phosphate Dehydrogenase

PBS- Phosphate Buffered Saline

DAPI- 4',6-Diamidino-2-Phenylindole

PHI- Peptide Histidine Isoleucine

Introduction

Vasoactive intestinal peptide (VIP) is an endogenous, 28 amino acid neuropeptide, with a broad array of biological functions both in the central nervous system and peripheral organs including the intestine (8, 46). It belongs to a superfamily of structurally related brain-gut peptide hormones (28). These peptides include important neuroendocrine mediators such as PACAP (Pituitary Adenylate Cyclase Activating Polypeptide) and intestinal hormones such as glucagon (39). In the gastro intestinal tract (GIT), VIP is known to regulate many physiological processes in different regions. For example, in the upper intestine it mainly mediates smooth muscle relaxation for peristaltic movement and sphincter functions (6, 54). VIP is also important in secretion of luminal ions and fluid in the pancreas and jejunum (11, 30) and is imperative as an enteric neuropeptide for tonic inhibitory control of the small intestinal circular muscle in the ileum (13, 20). In the colon, VIP has been shown to be involved in smooth muscle relaxation (23), ion transport (4, 42), mucus secretion (40) and in colon carcinoma cell models, VIP effects cellular biochemical processes such as glycogenolysis (45). Apart from these effects it also mediates epithelial regeneration (52) and tight junction barrier function (3, 38) in the intestinal epithelium. Furthermore, studies on VIP global knock out (KO) mice show severe abnormalities in the morphology and function of the GIT, validating the significance of VIP as a key enteric hormone (36).

The diverse physiological effects of VIP are mediated through binding of the peptide with the seven transmembrane G-protein coupled receptors. VIP binds first, specifically to the N-terminal of its receptor and then to the other domains which requires the entire 28 amino acid residues of the peptide (32, 49). The receptors for VIP are structurally related to the secretin/glucagon superfamily of receptors and are common to both VIP and PACAP (which

163 shares almost 70% amino acid homology to VIP) (25). These receptors have been
164 pharmacologically classified into two of which show higher affinity for VIP, termed VPAC1 and
165 VPAC2, and one low affinity receptor PAC1 (25, 32). Since VIP receptors are stimulatory or
166 type 2 GPCRs (Gs), the molecular pathways of VPAC receptor activation and subsequent effects
167 have been mainly attributed to increased intra-cellular cyclic AMP (cAMP) through adenylate
168 cyclase. Among the three receptors VPAC1 have been identified as the predominant receptor in
169 different organs such as the liver, lung, thyroid and reproductive organs (44).

170 With regard to intestinal epithelial cells, early studies conducted by Laburthe *et al* with
171 the aid of radiolabeled VIP binding showed high binding of VIP to the intestinal epithelial cells
172 (12, 47). It should be noted that the discovery and characterization of the specific GPCR's for
173 VIP and its classification was only conducted later and was not defined at the time these studies
174 were undertaken (25, 31). In addition, the sub cellular localization of these receptors in the
175 intestinal epithelial cells has also not been characterized. Thus, very limited knowledge is
176 available regarding the expression of VIP receptors in the gastro intestinal mucosa.

177 Given the pleiotropic effects of VIP across different regions of the GIT, the current study
178 investigated the expression of the known VIP receptors, along the length of the human and
179 mouse intestine. Our studies for the first time, demonstrate the relative abundance of all VIP
180 receptors from jejunum to the distal colon in mice and the human intestine. We show that in the
181 human and mice, the major receptor for VIP in the gut is VPAC1. In addition, our results
182 demonstrate localization of VPAC1 predominantly to the luminal membranes in the human and
183 mice colon tissue. This study, therefore, provides valuable insights into the potential importance
184 of the luminal enteric peptide VIP in health, and its potential to be utilized in targeting strategies

for VIP based nanomedicines to various regions of the intestine for treating gut disorders e.g. IBD.

Materials and Methods

Mice

Male, 6-8 weeks old C57BL/6 mice (n=8) were purchased from Jackson laboratories, (Bar Harbor, ME). Mice were euthanized with carbon dioxide inhalation followed by cervical dislocation prior to harvesting intestinal tissues. All animal studies performed were approved by the animal care committee of the University of Illinois at Chicago and Jesse Brown Veterans Affairs Medical Center (Chicago, IL).

Human specimens

Formalin fixed, paraffin embedded human colon sections from unaffected areas of the colon from IBD patients were kindly provided by the department of pathology, University of Illinois at Chicago and Jesse Brown VA Medical Center.

Real time PCR

RNA was isolated from mice jejunum, ileum, proximal and distal colon mucosal tissues with Qiagen RNeasy kits (Valencia, CA). Total human RNA from jejunum, ileum, ascending and sigmoid colon was purchased from BioChain institute Inc. (Newark, CA). Equal amounts of RNA were reverse transcribed and amplified using Brilliant SYBR green qPCR master mix kit (Stratagene, La Jolla, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control for each sample. Primers used are listed in **Table 1**.

VPAC1 antibody

Commercially available VPAC1 antibody was purchased from Thermofisher scientific (Waltham, MA, catalog no: PA3-113) raised in rabbit against a synthetic peptide corresponding to human VPAC1 receptor residues, T (438) RVSPGARRSSSFQAEVSLV (457). The antibody was validated by using peptide competition assay and confirmed for cross reactivity in mice.

Western blotting

Protein lysates from mice intestinal mucosal scrapings from jejunum, ileum, proximal and distal colons were extracted and 75 μg protein from each region was used to for western analysis as described earlier (37). Briefly, protein samples were prepared with 4X laemmli sample buffer (Bio-Rad, Hercules, CA) and boiled for 5 mins. Samples were then loaded to 10% Mini-Protean pre-cast gel (Biorad) and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in phosphate buffered saline (PBS) for 1 hour followed by incubation with anti-rabbit VPAC1 primary antibody at a ratio of 1:250 or anti-rabbit GAPDH (Sigma-Aldrich St. Louis, MO) antibody, at a ratio of 1: 10,000 in 1% milk in PBS overnight at 4^0C. The bound antibodies were detected by horseradish peroxidase-conjugated anti-rabbit Ig secondary antibody (Santa-Cruz, Santa Cruz, CA) followed by ECL detection system (Biorad) per manufacturer's instructions.

Peptide completion assay

Western analysis was performed for 75 μg of protein from Caco2 and mouse distal colon tissue as above and the nitrocellulose membranes were processed for primary antibody incubation as described below. Peptide corresponding to human VPAC1 receptor residues, T (438)

RVSPGARRSSSFQAEVSLV (457) was synthesized by pierce custom peptides (Termo fisher Scientific, Waltham, MA) and VPAC1 antibody (catalog number PA3-113) was purchased from Thermofisher Scientific. In an Eppendorf tube VPAC1 antibody was incubated at room temperature for 2 hours with or without the peptide at a 5 times excess. The resulting antibody mixtures were used in 1% milk and incubated overnight.

Immuno-fluorescence staining

Formalin fixed, paraffin embedded 5 μm sections from mice proximal and distal colon regions was stained as described previously with some modifications (22). Briefly, slides were placed at 60^0C for 30 min then immersed in xylene twice for 20 mins for deparafinization. The slides were then placed in a series of ethanol solutions (100, 95, 90,70 and 50%) in coupling jars for gradual rehydration. Afterwards the slides were immersed in distilled water for 5 mins. Antigen retrieval was performed by submerging the slides in a steam bath of 0.1M citrate buffer for 30 mins at 100^0C. Then the slides were allowed to cool to room temperature and rinsed in wash buffer (Tris- buffered saline containing 0.05% tween) for 5 mins followed by 5 min in permeabilization solution of tris- buffered saline containing 0.1% Triton X-100. The tissue sections were encircled with a water repellant pen and the slides were then incubated for an hour in a moist, dark container with 10% normal goat serum (NGS) to block non-specific antibody binding. This was followed by incubation of the slides with the primary antibody at a ratio of 1:100, antibodies used; VPAC1 (Thermofisher) and monoclonal anti-mouse villin (abcam, Cambridge, MA) or monoclonal anti-mouse actin (Sigma-Aldrich) or monoclonal anti- mouse Na^+ / K^+ ATPase (Thermofisher) 4^0C overnight in wash buffer containing 1% NGS. Following several washes, the slides were incubated with anti-goat secondary antibodies conjugated to either anti-rabbit Alexa

fluor 568 (red) or anti-mouse Aexa fluor 488 (green) (Thermofisher) for 1 hour at a ratio of 1:100 in wash buffer containing 1% NGS. After a few washes, the slides were mounted with prolong gold antifade / DAPI (Thermofisher) and sealed with clear nail polish. Slides were stored in -20^{0}C until use. Images were acquired with the use of the Olympus BX51 fluorescent microscope or Zeiss Axiocam acc1 (Oberkochen, Germany).

Statistical analysis

All data were subjected to One-way ANOVA (Tukey) or student's t-test (paired) statistical analysis and $P < 0.05$ or less was considered statistically significant.

Results

1.1 mRNA expression of VIP receptors in the mouse intestine

VIP is present abundantly in the intestine and mediates many important functions (28). Previous studies regarding the receptors for VIP in the intestine have been conducted only by radiolabeled iodine binding studies in rat, dog and humans. Additionally, there have been no studies conducted, to determine the expression of VIP receptors in the widely-used mouse model. Therefore, the current studies were undertaken to determine the specific receptor subtypes of VIP and their expression along the length of the intestine. As shown in Figure 1, when comparing the expression of individual receptors in regions from jejunum to distal colon, VPAC1 mRNA was found to be significantly higher in the colon as compared to the jejunum and ileum (**Figure 1A**) with similar levels in the proximal and distal colons (**Figure 1A**). VPAC2 mRNA distribution followed a pattern of Proximal colon > Distal colon > Jejunum = Ileum (**Figure 1B**). PAC1 receptor expression was also highest in the proximal colon, whereas there was almost no

difference in PAC1 levels among distal colon, ileum and jejunum (**Figure 1C**). PAC1 was also the least expressed receptor among the three receptors, along the length of the intestine. When comparing the expression of all receptors (VPAC1, VPAC2 and PAC1) along the intestine, the highest levels were observed in the colon followed by jejunum and ileum (**Figure 1D)**. In addition, VPAC1 mRNA was the most highly expressed receptor being almost 300-fold higher in all regions than VPAC2 and PAC1 mRNA. (**Figure 1D**). These results indicate that the predominant receptor of VIP, which may have the most functional relevance in the intestinal mucosa appears to be VPAC1.

1.2 VPAC1 Protein Expression along the length of the mouse intestine

The distribution of the mRNA levels clearly indicates that VPAC1 is the highly expressed receptor along the length of the mouse intestine. Therefore, to determine the protein levels of VPAC1 in regions of the intestine from jejunum to distal colon, western analysis was performed. A specific antibody (PA3-113) raised against VPAC1, was utilized to determine protein levels across regions. When the antibody was incubated with 5 times excess peptide, the band for VPAC1 at 55 kDa was significantly blocked (**Figure 2B**), demonstrating the specificity of the antibody in identifying both human and mouse VPAC1 receptor. In agreement with mRNA levels, the 55 kDa glycoprotein, VPAC1 was highly expressed in the proximal and distal colons ($p<0.05$) as compared to the jejunum and ileum (**Figure 2A**). Since the levels were highest in the colon, we next aimed to identify the cellular and membrane localization of the receptor VPAC1 in the colon.

2. Localization of VPAC1 receptor in mouse colon

In order to determine the cellular localization of the receptor, immunofluorescence staining with specific VPAC1 antibody was performed in mice colon, the region with highest expression of VPAC1. Interestingly, the VPAC1 receptor appeared to be localized to the apical membrane of the distal colon (**Figure 3A).** In the proximal colon, some sub-apical staining was also observed in addition to the predominant staining on the apical membrane (**Figure 3B**). To verify the apical localization, tissue sections were co-stained with the apical membrane marker, villin (**Figure 4**). The co-localization studies demonstrated that in the distal colon, the receptor was almost exclusively located to the apical membrane of the mucosa, as confirmed by co-localization with villin (green) (**Figure 4A**) and not to the basolateral marker Na^+/K^+ ATPase (green) (**Figure 4C**). Similarly, In the proximal colon, apical localization was confirmed by co-localization of VPAC1 (green) with villin (red) (**Figure 4B**). Villin was stained with two separate secondary antibodies in the proximal and distal colons to aid in differentiating between the two areas of the colon.

3. VPAC1 mRNA expression along the length of the human intestine

The results in mice demonstrated that the VPAC1 receptor is differentially expressed along the length of the mouse intestine. To determine if a similar pattern of expression is observed in the human intestine, we next performed qPCR analysis in total human RNA (BioChain) obtained from the human jejunum, ileum, ascending and sigmoid colons. In agreement with the mice data, VPAC1 expression was highest in the colon, more specifically in the human sigmoid colon (**Figure 5A**). However, in contrast to the data in mouse colon, where there was no difference between proximal and distal colons, expression of VPAC1 in the human sigmoid colon appeared to be markedly (~4 fold) higher compared to the ascending colon and small intestine. There was no difference in the expression levels of VPAC2 and PAC1 in various regions of the human

intestine (**Figure 5B, 5C**). These data indicated that VPAC1 was the predominant VIP receptor in the human intestine as well and its expression was relatively higher in the distal parts of the colon.

4. Localization of VPAC1 receptor in human colon sections

Since the mRNA expression showed highest level of VPAC1 in the human sigmoid colon, it was of interest to determine the membrane localization of VPAC1 in the human colon. Formalin fixed paraffin embedded 5 μm sections of human colons were stained for immunofluorescence studies with VPAC1 (red) and villin (green) antibodies. Similar to the results observed in mice, the localization of VPAC1 in the human colon was also predominantly restricted to the apical membranes (**Figure 6**).

Discussion

Abundantly present in the intestine, VIP is an important enteric peptide with an array of physiological functions (3). These biological effects are mediated via its binding to specific receptors, VPAC1, VPAC2 and less avidly to PAC1 (31). VPAC1 was first identified in the rat lung, and thereafter the human homolog was cloned and is considered to be the key receptor responsible for majority of VIP's biological functions (28). VPAC1 receptor is approximately a 55 kDa membrane bound protein present in many tissues such as the liver, lung, thyroid, central nervous system, immune cells and tumors (17, 28, 44).

The studies conducted herein provide for the first time, comprehensive data on the relative abundance of VIP receptors and its specific localization in the intestinal mucosa. Our results show comparable results to previous studies on the existence of VIP receptors in the

intestine (29, 33, 41, 47, 55). In addition, it addresses the gap in knowledge of the specific receptor sub-types of VIP in the intestinal mucosa and opens up investigation into the physiological relevance of these receptors. Earlier studies have attempted to determine the presence of VIP receptors in the gastrointestinal tract in the human, rat and dog. The main approach of these studies involved performing radiolabeled VIP binding in either isolated epithelial cells or parafinized tissue sections. In addition, at the time when these studies were undertaken, the specific sub-types of VIP receptors had not been characterized and the reagents for immuno detection were also limited. One key reason for lack of studies could be that after the recognition of anti-inflammatory characteristics of VIP, the major focus of VIP research shifted more to immune cells and non-GI organs (17, 30). It should also be noted that in the mouse model, the most widely used system *in vivo*, apart from one study conducted for VPAC2 receptor with radiolabeled ligand binding, there have been no studies conducted to determine the expression of VIP receptors in the intestine (26).

The key findings of our work are that the main receptor of VIP along the length of the mouse intestinal mucosa is VPAC1 and that the mRNA expression of VPAC1 is almost 300-times higher than the other receptors (**Figure 1**). In addition, the expression was higher in the colon as compared to jejunum and ileum. However, in the human, VPAC1 was mostly expressed in the sigmoid colon (~4 fold) compared to other regions and it was similar in the jejunum, ileum and ascending colon (**Figure 5**). The dissimilarity in expression pattern could be due to the RNA extracted from mucosal scrapings in mice and whole tissues in the human, this is potentially due to other VIP receptors being present in layers of the intestine from mucosa to muscle layers as compared to the mucosa alone. It could also be due to the difference in species. Protein data in mice also showed similar results to mRNA with higher levels of VPAC1 in the

colon (**Figure 2A**). The most intriguing result from this study was the atypical, apical localization of VPAC1 in the colonic epithelium (**Figures 3,4 and 6**). This is in contrast to previous studies, where radiolabeled VIP binding was indicated to be predominant in the basolateral membranes in the rat jejunum and rabbit ileum (18). In addition, several studies where effects of VIP were investigated in human colon carcinoma cell lines (Caco2 and HT29), VIP treatment was given basolaterally implying the presence of the receptors in the basolateral membrane (2, 19, 51). However, none of these studies provide conclusive data about the specific localization of VPAC1 in intestinal epithelial cells.

This controversial finding of VPAC1 localization led us to undertake antibody authentication experiments, which would aid in validating our findings. This specific antibody raised in rabbit (PA3-113) have been used by other investigators in human and rodent models (5, 21, 48). However, to determine the specificity of the antibody in recognizing the protein of interest in human and mice tissues, we performed peptide competition assay (**Figure 2B**). The blocking of the specific band with the addition of the specific peptide in both human and mouse tissue indicated to us that the detection of the protein was accurate. In addition, we used tissues from mice known to express less VPAC1 (kidney) as a negative control (53) and validated the results by performing qPCR on RNA and then confirmed this finding by western analysis showing comparable results (data not shown).

Mucosal lining of the colon plays a vital role in fluid reabsorption and its impairment leads to pathological conditions which are predominant in the distal parts of the intestine, such as ulcerative colitis (35). Abundance of VPAC1 in the colon could therefore, play a role under these conditions. Previous studies have provided valuable hints to the possible existence of VIP receptors in the epithelial cells and its potential presence on the apical membrane. Namely the

radio-iodinated VIP binding studies in human colon sections demonstrated specific high binding of radiolabeled VIP to the colonic mucosa as compared to other parts in tissue cross sections, indicating existence of VIP binding through receptors to the mucosa (55). In addition, multiple studies performed by Laburthe *et al.* utilizing radiolabeled VIP indicated the existence of VIP receptors on epithelial cells isolated from intestinal mucosa from rats and humans (7, 41, 47). However, these studies were conducted in cell suspensions isolated from human and rat intestines and the term receptor was used to describe the specific binding of VIP to cells rather than identifying the target receptor protein itself (7, 41). With the advent of specific receptor sub-type identification, another previous study utilized a radiolabeled analog of VIP with higher affinity for VPAC2 receptor (VPAC2 agonist) and showed minimal binding in the colonic epithelium (26). This is consistent with our current data demonstrating a significantly less expression of VPAC2 as compared to VPAC1 in the colon.

The most important question arising from the presence of VPAC1 receptors in the luminal membrane of the intestine is its physiological relevance. Enteric neurons are known to secrete neuropeptides such as VIP into the close proximity of the intestinal lumen, due to the existence of nerve bodies in capillaries present in the mucosa (10). Another group have demonstrated that VIP is found in luminal contents after being stimulated (9). These studies provide a sound indication of potential physiological functions of luminal VIP in the intestine. Since VIP only mediates functions by binding to its receptors, luminal localization of VPAC1 may directly partake in mediation of these various functions.

The abundance and localization of VPAC1 in colon of mice and humans may indicate that some of the key functions of VIP such as affecting epithelial regeneration (24), ion transport (42), fluid secretion (27), mucus secretion (40) and tight junction protein expression (38) can be

directly mediated through the luminal receptors. The colonic epithelium undergoes constant regeneration due to being exposed to solid fecal contents which passes through the lumen. Furthermore, the very minimal presence of fluid in the colon requires mucus secreted to the lumen so that avid lubrication could allow rapid passage of the luminal contents. VIP affects epithelial regeneration and mucus secretion in mucosa including the intestine which could indicate that VIP may affect these parameters in the colon. Apart from VIP, the receptor VPAC1 has lower affinity to other related peptides such as secretin and peptide histidine isoleucine (PHI) and thus may also serve as a luminal signaling sensor of these secreted peptides.

The specific apical localization of VPAC1 also widens possibilities to target neuropeptide therapies to the colon, once administered luminally. Previously, we and others have used VIP receptors as a targeting modality to various cancer cell populations (14-16, 43). Being an immunomodulatory agent, VIP can also be successfully used to ameliorate colitis (1). If therapies with VIP can be administered via oral route, the apical receptors would directly participate in the mediation of the anti-inflammatory and regenerative effects of VIP in reducing the disease pathology. The abundance of VPAC1 receptors in the lumen of the colon can be utilized to deliver VIP in drug delivery systems which resist the acidity of the stomach, and are preferentially released in the colon. This can be achieved either by using enteric coated capsules (50) or by hydrogel systems (34) with VIP as an active agent so that release will take place specifically in the colonic lumen once administered orally.

Figure captions

Figure 1: VIP receptor mRNA expression along the length of the mouse intestine

mRNA isolated from mouse intestinal mucosa from jejunum, ileum, proximal and distal colon were subjected to qPCR with specific priers for VIP receptors: VPAC1, VAPC2 and PAC1. **A)** Graphical representation of VPAC1, **B)** VPAC2, **C)** PAC1 receptors expression normalized to internal control GAPDH; expression of receptors were compared to its expression in jejunum (arbitrary units). **D)** compiled data of all receptor expression where PAC1 expression in the jejunum (arbitrary units). Values represent mean ± SEM, n=6, *p<0.05 Vs jejunum and ileum, ***p<0.0005 Vs jejunum and ileum.****p<0.0001 Vs jejunum and ileum, ####p<0.0001 Vs distal colon.

Figure 2: VPAC1 protein expression along the length of the mouse intestine

A) Representative western blot and graphical representation of densitometric analysis of VPAC1 protein level expressed in mucosal protein extracted from jejunum, ileum, proximal and distal colons of the mouse. VPAC1 protein was detected at 55 kDa. Higher expression was observed in both proximal and distal colons. Values represent mean ± SEM, n= 8, *p<0.05 Vs jejunum **B)** Antibody- peptide competition assay in caco2 and mouse distal colon protein lysates. Data shows specificity of VPAC1 band when antibody was incubated with 5 times excess of peptide.

Figure 3: VAPC1 expression in mouse proximal and distal colon

Representative images of formalin fixed paraffin embedded tissue sections from mouse **A)** distal and **B)** proximal colons of 5 μm stained with VPAC1 (alexa fluor 568, red) and actin (alexa

fluor 488, green) and mounted with DAPI (blue) staining nuclei. Red staining was found predominantly in the luminal membrane in the distal colon with some sub-apical staining present in the proximal colon.

Figure 4: Apical localization of VPAC1 in the mouse colon

Representative images of formalin fixed paraffin embedded 5 μm tissue sections from mouse **A)** distal colon stained with VPAC1 (alexa fluor 568, red) and villin (alexa fluor 488, green) **B)** proximal colon stainied with VPAC1 (alexa fluor 488, green) and villin (alexa fluor 568, red) mounted with DAPI (blue) staining nuclei. Yellow staining demonstrated co-localization of VPAC1 with apical marker villin. **C)** distal colon sections were stained separately with Na^+ / K^+ ATPase (alexa fluor 488, green) or VPAC1 (alexa fluor 568, red) and DAPI confirming apical localization. Separate staining of VPAC1 and Na^+ / K^+ ATPase was performed due to non-compatibility of the primary antibodies when stained together.

Figure 5: VIP receptor mRNA expression along the length of the human intestine

Total mRNA isolated from human jejunum, ileum, proximal and distal colons were subjected to qPCR with specific priers for VIP receptors: VPAC1, VAPC2 and PAC1. **A)** Graphical representation of VPAC1, **B)** VPAC2 and **C)** PAC1 receptor expression normalized to internal control GAPDH; expression of receptors was compared to its expression in jejunum where the expression was set to 1. **D)** Compilation of VIP receptor mRNA expression in all regions where the expression in the jejunum was set to 1.

Figure 6: Apical localization of VPAC1 in human colon tissue

Representative image of formalin fixed paraffin embedded 5 μm section of the human colon stained with VPAC1 (alexa fluor 568, red) and villin (alexa fluor 488, green) and mounted with DAPI (blue) staining nuclei. Yellow indicates co-localization of VPAC1 and villin confirming apical localization. This representative image is from an unaffected colon of a 25-year old male patient.

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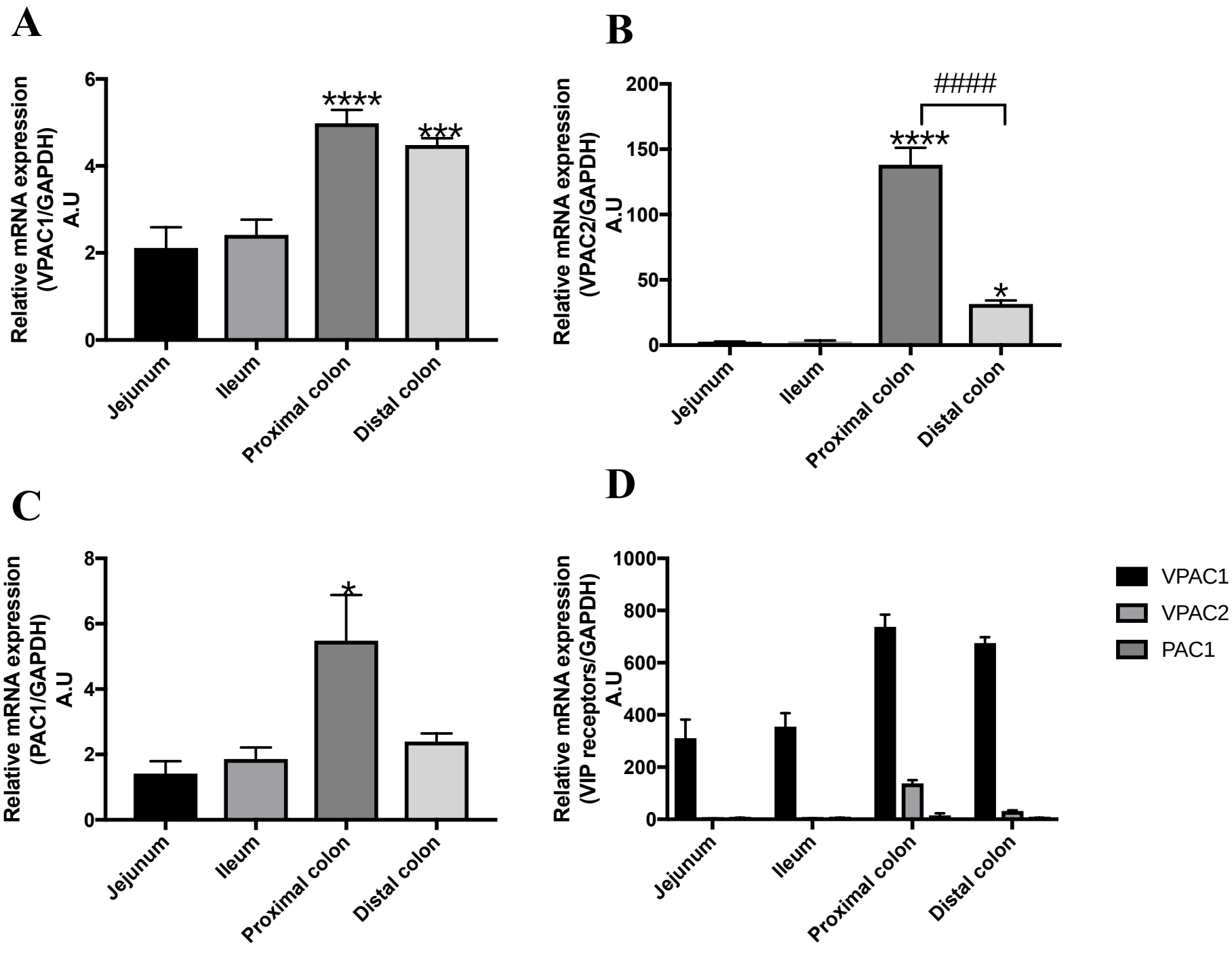
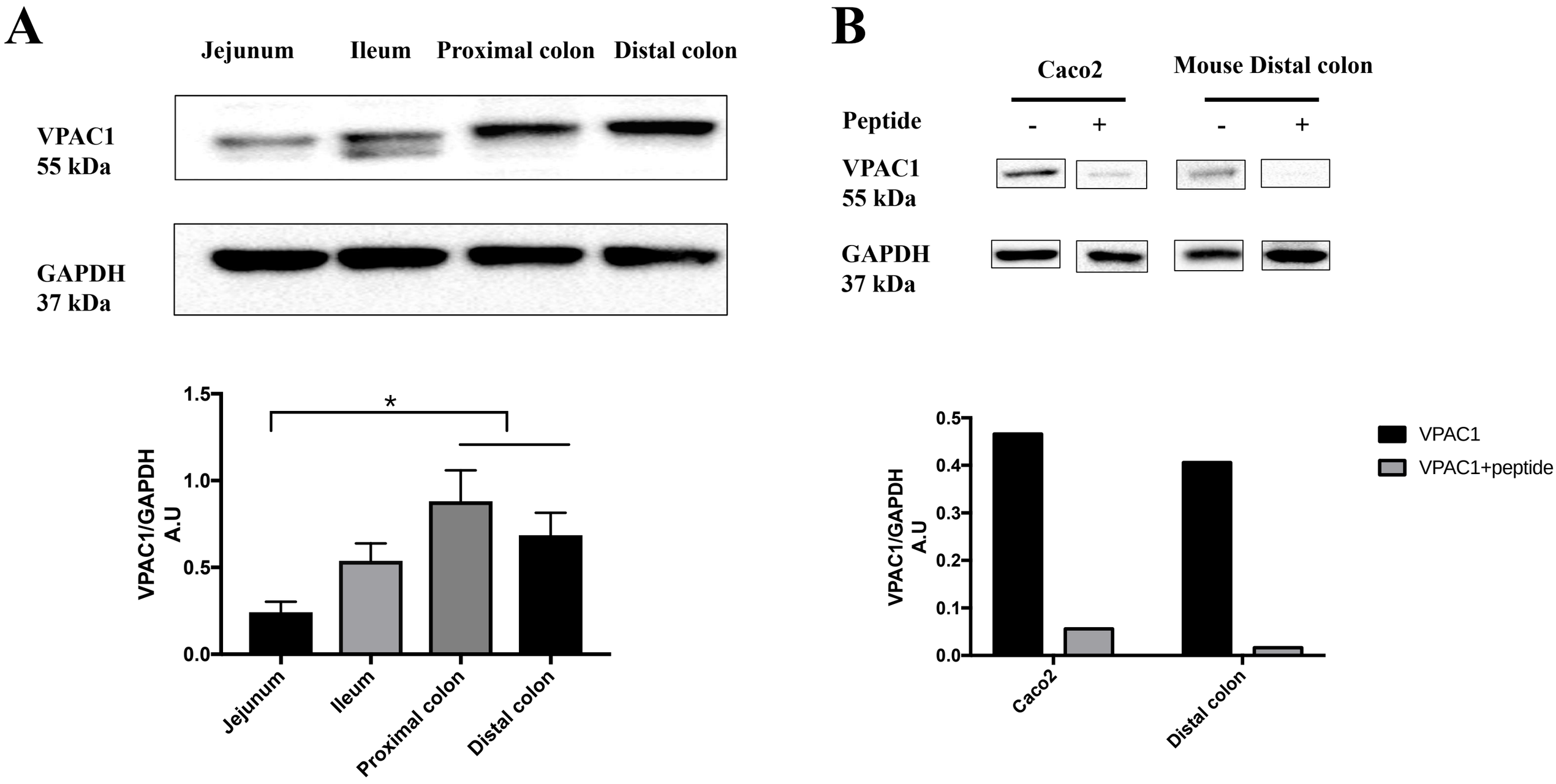
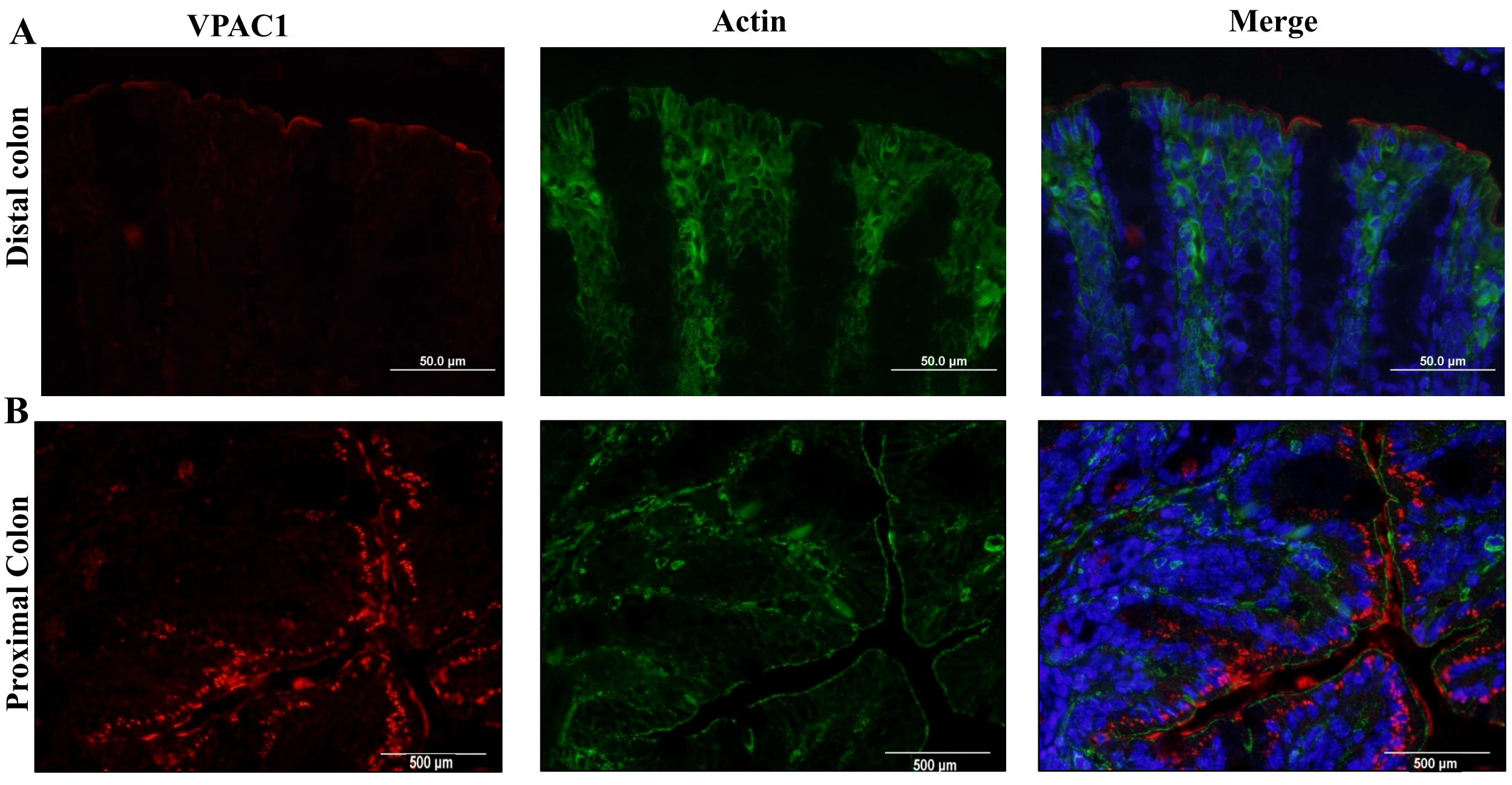
Table 1: Gene specific primer sequences

Gene	Sequence (5' → 3')	Gene Accession no
Human VPAC1	F-TCATCCGAATCCTGCTTCAGA R- AGGCGAACATGATGTAGTGTACT	NM_001251884
Mouse VPAC1	F- GATGTGGGACAACCTCACCTG R- TAGCCGTGAATGGGGGAAAAC	NM_011703
Human VPAC2	F-CAGTGGCGTCTGGGACAAC R- CCGTCACTCGTACAGTTTTTGC	NM_003382
Mouse VPAC2	F- GGTGAGCAGCATCCATCCAG R- TCGCTAGTGCAGTTTTTGCTTA	NM_009511
Human PAC1	F-GTCGGAACCCTTCCCTCATTA R-GGCCTTCACTGACAGGTAGTA	NM_001199635.1
Mouse PAC1	F-GGCTGTGCTGAGGCTCTACTTTG R-AGGATGATGATGATGCCGATGA	NM_007407.4
Human GAPDH	F-GAAATCCCATCACCATCTT R-AAATGAGCCCCAGCCTTCT	NM_002046.5
Mouse GAPDH	F-TGTGTCCGTCGTGGATCTGA R-CCTGCTTCACCACCTTCTTGAT	NM_001289726.1


A
Relative mRNA expression (VPAC1/GAPDH) A.U
6
4
2
0
****
***
Jejunum
Ileum
Proximal colon
Distal colon
B
Relative mRNA expression (VPAC2/GAPDH) A.U
200
150
100
50
0
#####
****
*
Jejunum
Ileum
Proximal colon
Distal colon
C
Relative mRNA expression (PAC1/GAPDH) A.U
8
6
4
2
0
*
Jejunum
Ileum
Proximal colon
Distal colon
D
Relative mRNA expression (VIP receptors/GAPDH) A.U
1000
800
600
400
200
0
Jejunum
Ileum
Proximal colon
Distal colon
VPAC1
VPAC2
PAC1


A
Jejunum
Ileum
Proximal colon
Distal colon
VPAC1
55 kDa
GAPDH
37 kDa
VPAC1/GAPDH
A.U
1.5
1.0
0.5
0.0
*
Jejunum
Ileum
Proximal colon
Distal colon
B
Caco2
Mouse Distal colon
Peptide
-
+
-
+
VPAC1
55 kDa
GAPDH
37 kDa
VPAC1/GAPDH
A.U
0.5
0.4
0.3
0.2
0.1
0.0
VPAC1
VPAC1+peptide
Caco2
Distal colon


A
VPAC1
Actin
Merge
Distal colon
50.0 µm
50.0 µm
50.0 µm
B
Proximal Colon
500 µm
500 µm
500 µm

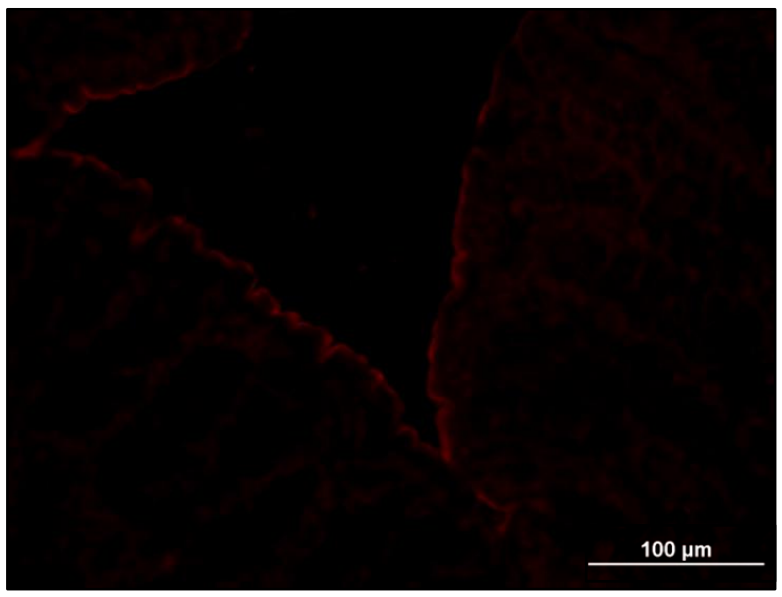
A

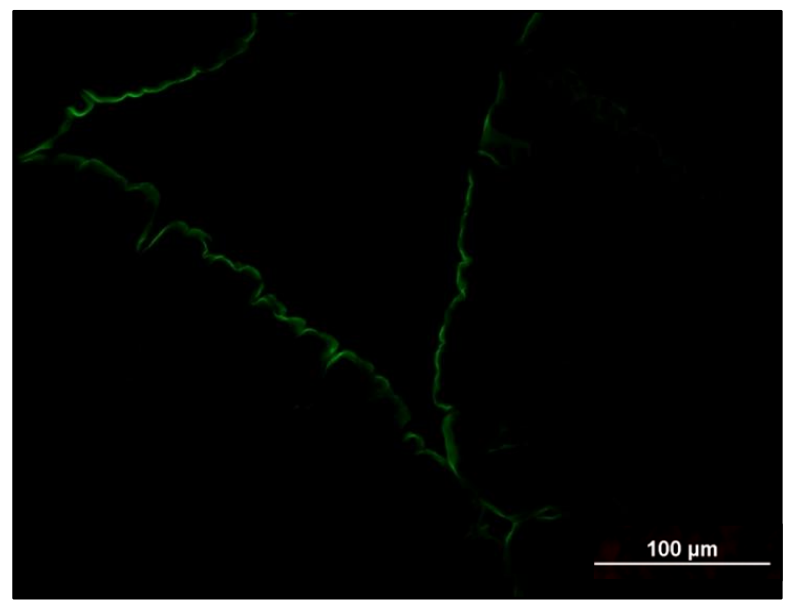
VPAC1

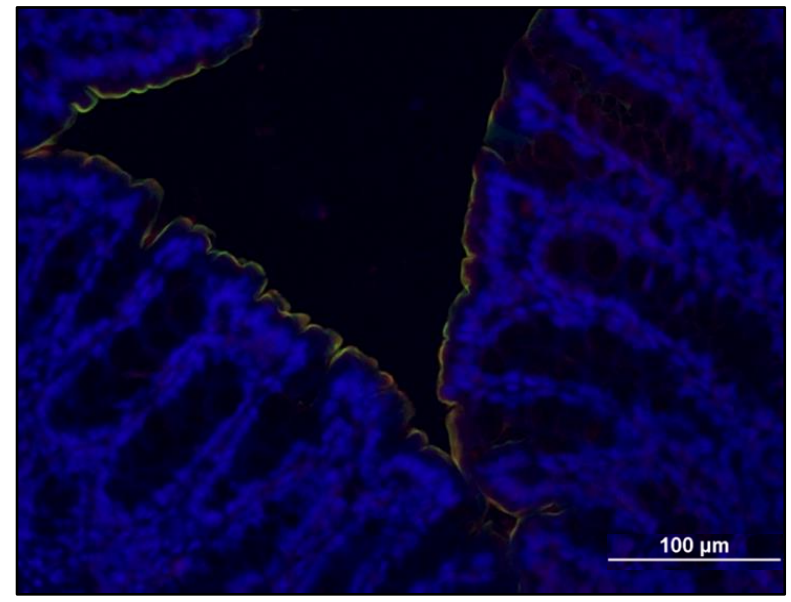
Villin

Merge

Distal colon

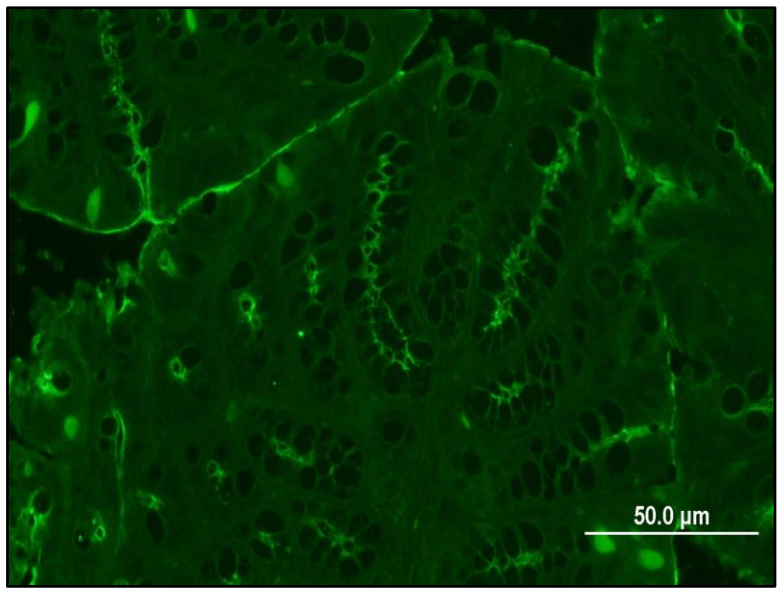


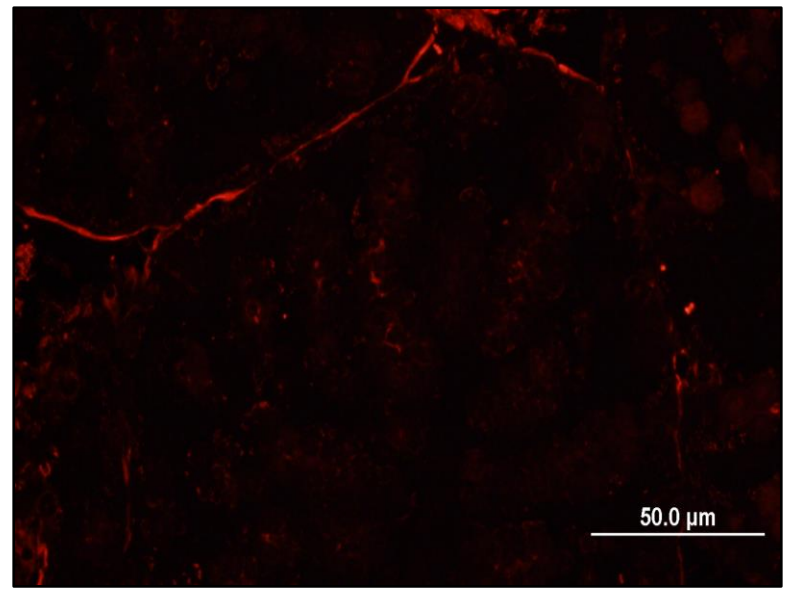


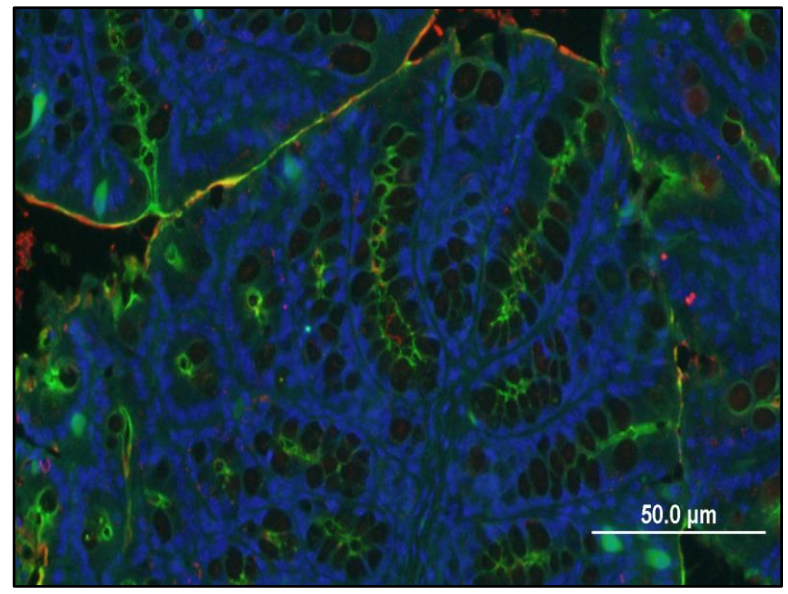


B

Proximal Colon



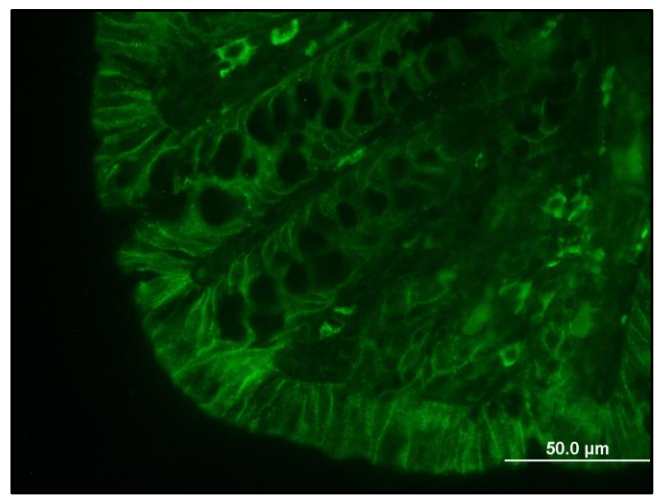


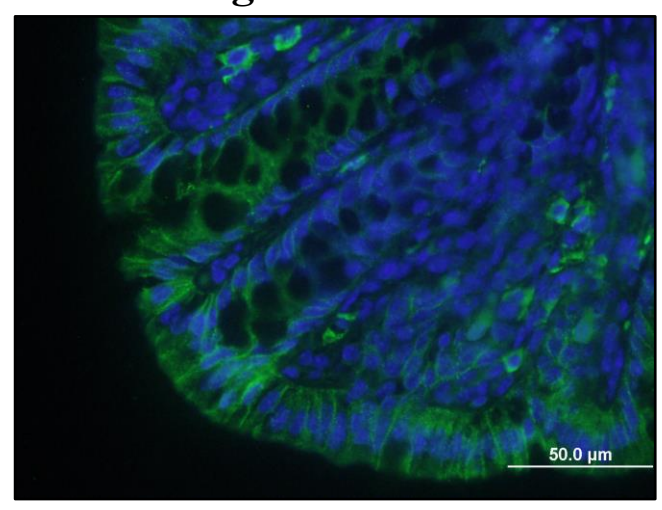


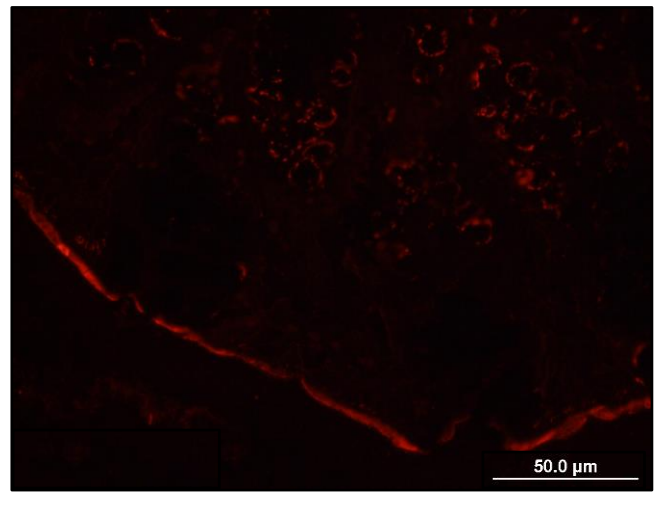
C

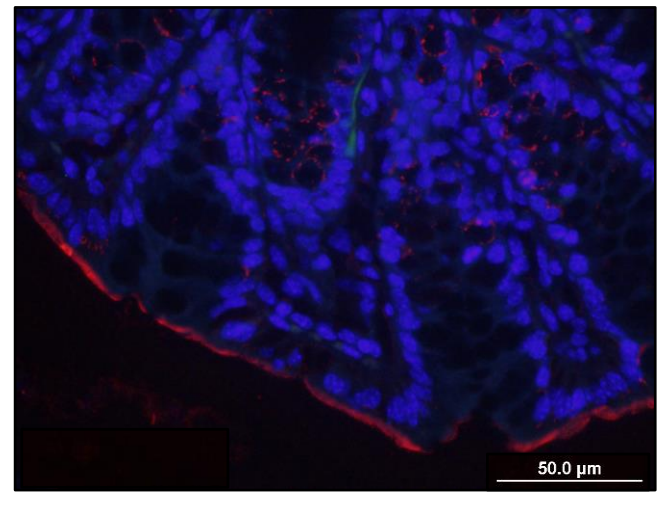
Na^+ / K^+ ATPase

Merge/ with DAPI



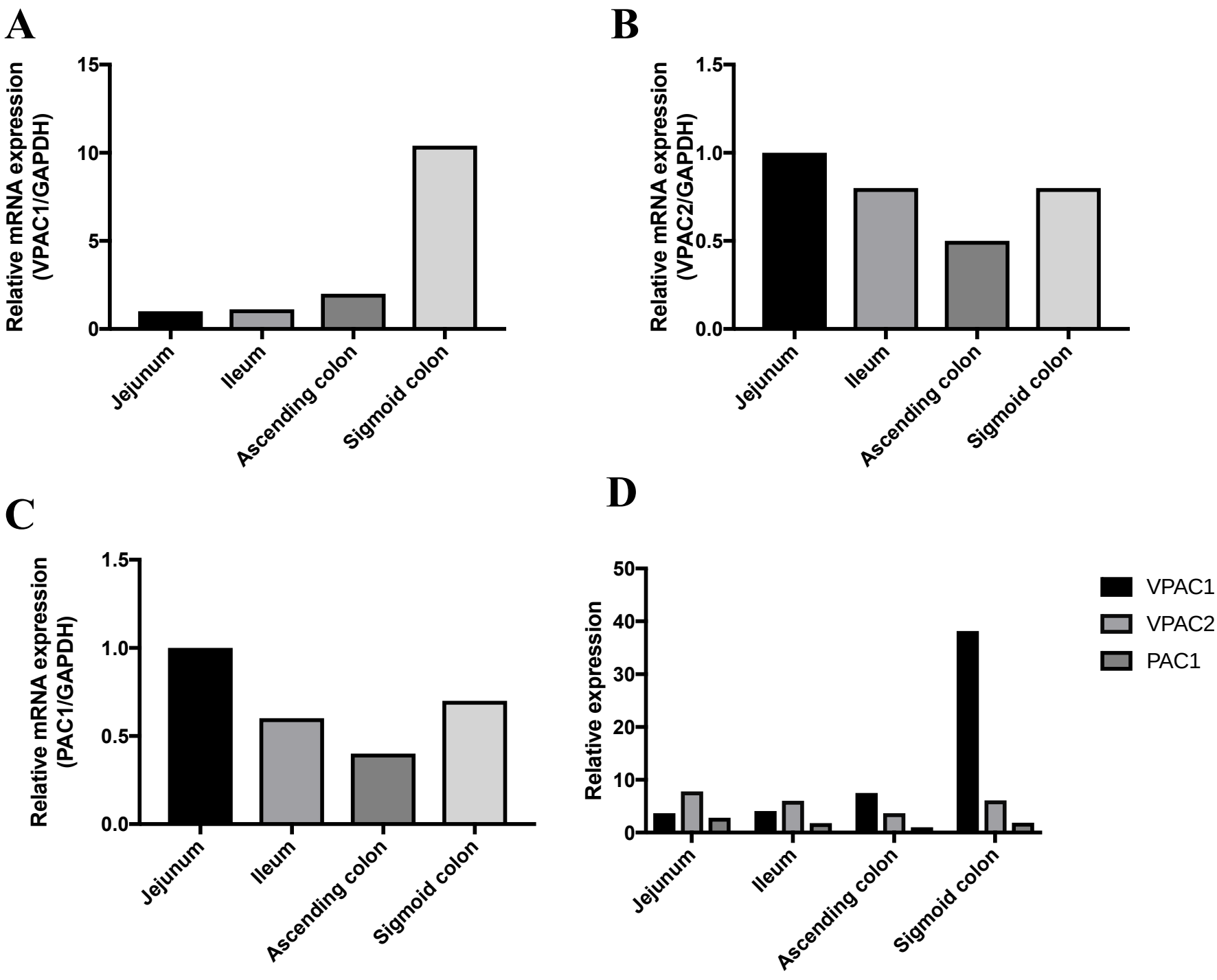




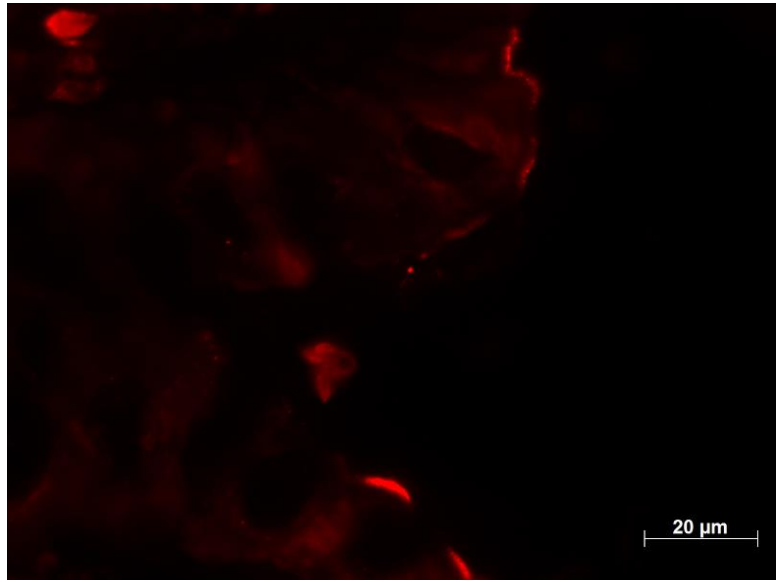


VPAC1

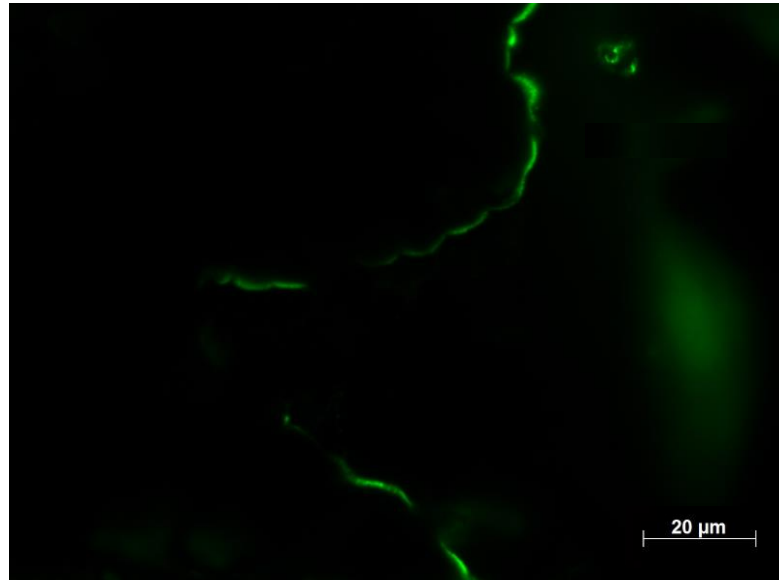
Merge/ with DAPI


A
Relative mRNA expression (VPAC1/GAPDH)
15
10
5
0
Jejunum
Ileum
Ascending colon
Sigmoid colon
B
Relative mRNA expression (VPAC2/GAPDH)
1.5
1.0
0.5
0.0
Jejunum
Ileum
Ascending colon
Sigmoid colon
C
Relative mRNA expression (PAC1/GAPDH)
1.5
1.0
0.5
0.0
Jejunum
Ileum
Ascending colon
Sigmoid colon
D
Relative expression
50
40
30
20
10
0
Jejunum
Ileum
Ascending colon
Sigmoid colon
VPAC1
VPAC2
PAC1

VPAC1



Villin



Merge