Ano1 is a Selective Marker of Interstitial Cells of Cajal in the Human and Mouse Gastrointestinal Tract

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Short Title: Ano1 is a marker for ICC

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ABSTRACT (250 word limit)

Populations of interstitial cells of Cajal (ICC) are altered in several gastrointestinal neuromuscular disorders. ICC are identified typically by ultrastructure and expression of Kit (CD117) a protein that is also expressed on mast cells. No other molecular marker currently exists to independently identify ICC. The expression of ANO1 (DOG1, TMEM16A), a Ca$^{2+}$-activated Cl$^{-}$ channel, in gastrointestinal stromal tumors suggests it may be useful as an ICC marker. The aims of this study were therefore to determine the distribution of Ano1-immunoreactivity compared to Kit and to establish whether Ano1 is a reliable marker for human and mouse ICC. Expression of Ano1 in human and mouse stomach, small intestine, and colon was investigated by immunofluorescence-labeling using antibodies to Ano1 alone and in combination with antibodies to Kit. Colocalization of immunoreactivity was demonstrated by epifluorescence and confocal microscopy. In the muscularis propria, Ano1-immunoreactivity was restricted to cells with the morphology and distribution of ICC. All Ano1-positive cells in the muscularis propria were also Kit-positive. Kit-expressing mast cells were not Ano1-positive. Some non-ICC in the mucosa and submucosa of human tissues were Ano1-positive but Kit-negative. A few (3.2%) Ano1-positive cells in the human gastric muscularis propria were labeled weakly for Kit. Ano1 labels all classes of ICC and represents a highly specific marker for studying the distribution of ICC in mouse and human tissues with an advantage over Kit as it does not label mast cells.

Keywords: Kit, mast cells, Chloride channels, Gastrointestinal motility, immunofluorescence
INTRODUCTION

Interstitial cells of Cajal (ICC) are mesoderm-derived mesenchymal cells that contribute to normal gastrointestinal motility (9). ICC generate pacemaker potentials that drive the electrical slow wave (19, 36) contribute to normal neuromuscular signaling (4), are involved in mechanotransduction (13, 31) and set gradients in smooth muscle membrane potential (10).

ICC were originally identified by Cajal and were characterized using morphologic criteria until the discovery that these cells express the receptor tyrosine kinase Kit (23). Subsequent studies determined that Kit-immunoreactivity in the muscularis propria of the gastrointestinal tract is restricted to two cell types, mast cells (9, 25) and ICC. Kit-positive ICC are distributed throughout the gastrointestinal tract as well as in other smooth muscle tissues (24). All regions of the gastrointestinal tract contain ICC but the location within the muscularis propria varies according to region or species (15). In the myenteric plexus region, ICC form a network between the muscle layers forming a mesh around the ganglia (ICC-MY). A network of deep muscular plexus ICC (ICC-DMP) is present in the small intestine between the inner and outer circular muscle layers (15). In the colon and parts of the gastric antrum, sub-muscular ICC (ICC-SM) are located outside the circular muscle layer (2, 3). Intramuscular ICC (ICC-IM) are distributed through the longitudinal and circular muscle layers. Septal ICC are found between the fascicles of muscle in humans and other large species. These can be considered a type of ICC-IM (29, 37). Stellate, sub-serosal ICC are observed on the boundary between the longitudinal muscle and the serosa in the colon of mice (34).
At present, Kit is the only reliable antigenic marker for ICC. Antibodies to Kit have been used extensively to characterize changes in ICC networks in human and animal tissue. Several human gastrointestinal motility disorders have been associated with depletion of Kit-positive ICC (11, 12, 16, 22, 26), however these observations have not been confirmed by using antigenic markers independent of Kit signaling. Also, residual ICC-like function in ICC deficient mutants has been linked to Kit-negative cells (35). These cells may be related to ICC and may be revealed by a Kit independent marker. Advances in gene expression profiling have been applied to identifying markers for ICC (6, 40) resulting in the demonstration that the Na⁺/K⁺/2Cl⁻ co-transporter, NKCC1 (39), the neurokinin-1 receptor (7) and CD44 (21) are all proteins expressed selectively on some or all sub-types of ICC. At present, no comprehensive survey of the gastrointestinal tract has been published using antisera to any of these targets.

One protein identified recently as expressed on ICC is Ano1 (previously known as FLJ10261, DOG-1 and TMEM16A) (8, 38). Ano1 is part of a family of 10 gene products (Ano1-Ano10 or TMEM16A to TMEM16K) with similar primary sequences and predicted secondary structures (20, 41). Ano1 expression is up-regulated in gastrointestinal stromal tumors (8, 38) and other tumors (20). In non-transformed cells, Ano1 is expressed in many organs including epithelia from the lung, foregut (28), and kidney, as well as pancreas and salivary glands (41). Knockout of Ano1 leads to death of the pups early after birth (28). Expression studies have determined that the cloned Ano1 gene product contributes to a Ca²⁺-activated Cl⁻ conductance (5, 30, 41).

ICC in human colon and small intestine also appear to express Ano1 (8, 14, 38). It is not known whether Ano1 is expressed in all regions of the gastrointestinal tract,
whether all types of ICC express Ano1, if all Kit-positive ICC express Ano1 and vice versa, or if Ano1 is expressed on mouse ICC. Therefore, the objective of this study was to investigate the potential utility of Ano1 as a Kit-independent marker of ICC by examining the distribution of Ano1-positive cells in the gastrointestinal tract of human and mouse tissue and determine the degree of colocalization of Ano1 with Kit on ICC.

MATERIALS AND METHODS

Tissues. Human gastric mid-body tissues (n=3) and jejunal tissues (n=3) were obtained from 6 patients undergoing surgery for morbid obesity. Normal human colon (n=3) was obtained from 3 patients undergoing resection for non-obstructing colon cancer (details in table 1). Tissues were placed in ice cold F12 medium (Invitrogen, Carlsbad, CA). A piece of tissue 2 cm x 2 cm was dissected, pinned out, flash frozen in isopentane cooled with dry ice and frozen in OCT embedding compound (Sakura Finetek USA Inc, Torrance, CA). Fresh frozen tissues were stored at -80°C until sectioned.

Adult BALB/c mice (4-8 week old; n = 6) were purchased from Harlan Laboratories (Madison, WI). The animals were anesthetized by isoflurane (Aerrane; Baxter Healthcare, Deerfield, IL) inhalation and killed by decapitation. Mouse tissues (gastric fundus, gastric corpus and antrum, jejunum and ileum, proximal colon, distal colon) were excised, placed in ice-cold Krebs–Ringer bicarbonate buffer (21) and opened along the lesser curvature of the stomach or the insertion of the mesentery and their contents were washed away with ice-cold Krebs–Ringer bicarbonate buffer. The mucosa and submucosa were removed by peeling and only the muscularis propria was used for immuno-labeling experiments.
Antibodies. For detail about the primary and secondary antisera used see table 2. The specificity of rabbit Ano1 antibody was demonstrated by the absence of labeling in colon tissue from Ano1 knockout mice when compared to wild type animals provided by Dr Brian Harfe, University of Florida (27, 28).

Controls for each antibody used were carried out by incubating the sections with secondary antibodies but no primary antibodies, by applying secondary antibody directed against IgG from a species that was not the host for raising the primary antibody and by examining singly labeled tissues under illumination with the filter sets designed for the wrong fluorophore.

Immuno-labeling. Human tissues were cut in 12 μm thick sections while the immuno-labeling for mice tissues were carried out in whole-mounts devoid of mucosa and submucosa. Tissues from human were fixed in 25% acetic acid/75% ethanol (v/v) solution for 10 minutes. After blocking for 2 hours at room temperature in 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) in PBS the sections were incubated overnight at 4°C with the primary antibodies for Kit and Ano1 (see supplementary Table 2) in 0.3% (v/v) Triton X-100 plus 1% BSA in PBS. After washing, the tissue was incubated for 1 hour with the appropriate secondary antibodies (see Table 2), washed and counterstained with 4’,6-diamidino-2-phenylindole dilactate (DAPI dilactate, Invitrogen, Carlsbad, CA) to label nuclei. The murine whole-mounts were stretched over the surface of a Sylgard 184 (Dow Corning, Midland, MI)-coated Petri-dish, fixed with cold acetone (4°C, 10 min), washed with PBS (4°C, overnight) and blocked with 1% BSA (1 hour at room temperature). The tissues were then incubated for 48 hours at 4°C with a rat monoclonal anti-murine Kit antibody (ACK2; see Table 2) in 0.3% (v/v) Triton X-100
plus 1% BSA in PBS. After a second fixation step with 4% paraformaldehyde-PBS (10 min at room temperature), the tissues were washed with cold PBS overnight, blocked again with 1% BSA in PBS for 1 hour and labeled with the rabbit polyclonal anti-Ano1 also used in human tissues (48 hours at 4°C in PBS containing 1% BSA). Secondary antisera (Table 2) were applied after an overnight wash with PBS at room temperature for 1 hr. To label mast cells, using an antibody to mast cell tryptase, antigen retrieval was required. Human colon was fixed overnight in 4% paraformaldehyde and the next day the tissue was washed and then incubated overnight in 30% sucrose. Twelve μm sections were cut and subjected to antigen retrieval. This was carried out using the steamer method and using preheated antigen retrieval solution (Dako, Carpinteria, CA, USA) at 90–96°C. The slides were maintained at this temperature for 15 minutes. After antigen retrieval, tissues were blocking for 2 hours at room temperature in 1% BSA and incubated overnight at 4°C with the primary antibodies for mast cells tryptase and Ano1 (see supplementary Table 2) in 0.3% (v/v) Triton X-100 plus 1% BSA in PBS. The next day, the tissue was incubated for 1 hour with the appropriate secondary antibodies (see Table 2), washed and counterstained with 4’,6-diamidino-2-phenylindole dilactate (DAPI dilactate, Invitrogen, Carlsbad, CA) to label nuclei.

*Image acquisition.* Three to ten sections from each tissue were examined using an Olympus BX51WI epifluorescence microscope (Olympus America Inc, Center Valley, PA, USA) or by confocal microscopy (Olympus FV300, Melville, NY). The confocal images were collected using the optimal pinhole size for the 60X, 1.2 NA water objective at 633 nm (z axis step 0.48 μm; human tissues) or for the 40X, 1.0 NA oil objective at 543 nm (z axis step: 0.7 μm; mouse tissues). The following lasers and emission filters
were used to visualize the labeled structures and collect images: multi-line Ar laser at 488 nm (used for the excitation of Alexa Fluor 488); emission filter: 535±15 nm; 543 nm HeNe laser (used for Cy3 and Alexa Fluor 594); emission filter: 575-630 nm; and 633 nm HeNe laser (used for Cy5); emission filter: HQ660 nm (650-700 nm).

Quantification of Ano1-positive cells in human stomach. The numbers of Ano1 and Kit-positive cells in the circular muscle layer of the human stomach tissue were counted at 200X magnification. Cells in 30 fields from three non-adjacent sections were counted (10 fields per slide). Ano1-positive cells were scored as either brightly Kit-positive or weakly Kit-positive (Kit-dim). Kit-positive cells were scored as either Ano1-positive or Ano1-negative.

Materials. Unless indicated, reagents were from Sigma-Aldrich (St Louis, MO).

RESULTS

Ano1-positive cells in the human gastrointestinal tract. In the human gastrointestinal tract (Fig. 1), Ano1-immunoreactive cells were detected using both the polyclonal rabbit antiserum (Abcam) and the monoclonal mouse antiserum (Applied Genomics) by labeling of acid-ethanol fixed sections. Use of other fixation conditions, including 2-4% paraformaldehyde (with and without antigen retrieval) and cold acetone, resulted in non-specific labeling of cell nuclei and much dimmer signal. Double labeling with the two antibodies to Ano1 resulted in exactly coincident signals (Fig. 1A).

The Ano1-positive cells detected in the muscularis propria had the characteristic distribution pattern of ICC for each region. All types of ICC identified previously by Kit-immunoreactivity (15) were identified by Ano1-immunoreactivity. In the body of the stomach, Ano1-positive cells were present in both the longitudinal and circular muscle
layers as well as in the region of the myenteric plexus and in the submucosal plexus (Fig. 1B). As reported for Kit-positive ICC (32), myenteric networks of Ano1-positive cells were considerably less dense in the stomach than in other tissues. Ano1-positive cells were also observed in septa. In the human jejunum, Ano1-positive cells were observed in the myenteric plexus region, in septa, and in the DMP (Fig. 1C). Ano1-positive, ICC-like cells in the human colon were present in the longitudinal and circular muscle layers including septa, the myenteric plexus region, and the submucosal plexus (Fig. 1D).

The morphology of the Ano1-positive cells in the muscularis propria of the tissues was characteristic of ICC. Cells within the muscle layers were oriented parallel to the long axis of the myocytes and had branching processes running in opposite directions from the cell body (Fig. 2A). In the septa, the Ano1-positive cells were located between the muscle bundles (Fig. 2B). Cells in the myenteric plexus regions were located around the periphery of the ganglia forming a dense network of Ano1-positive processes. These ICC-MY-like cells had triangular cell bodies and two or more processes (Fig. 2C). In the DMP, the Ano1-positive cells had bipolar morphology and two processes running parallel to the circular muscle layer (Fig. 2D). Ano1-positive cells characteristic of ICC-SM were observed close to the circular muscle in the sub-mucosa of the colon and stomach. These cells were also predominantly bipolar in morphology (Fig. 2E).

Ano1-positive cells with a different morphology from ICC were also detected in areas that did not contain Kit-positive ICC. Specifically, Ano1-positive cells were located in the mucosa and sub-mucosa of all regions studied (Fig. 2F).

**Colocalization of Kit and Ano1.** To confirm the identity of the Ano1-positive cells as ICC, tissue was co-labeled for both Ano1 and Kit. The rabbit polyclonal antiserum to Kit
labeled the ICC-like cells identified using the mouse monoclonal antibody to Ano1. Similarly, the mouse monoclonal antiserum to Kit labeled the ICC-like cells identified using rabbit polyclonal antiserum to Ano1 (Fig. 3A and 3B). All Ano1-positive cells in the external muscle layers were also Kit-positive in all regions of the gastrointestinal tract (Fig. 3C-E) and the co-localization of Ano1 and Kit was complete within the spatial resolution of confocal microscopy (Fig. 3F). There was no labeling of ICC-like cells by any of the secondary antibodies in the absence of primary antiserum. There was also no labeling of ICC-like cells when the anti-mouse secondary antisera were applied to tissues incubated with the primary antisera raised in rabbit or vice-versa. No signal was observed when singly labeled tissues were examined using the excitation and emission filters for the wrong fluorophore.

The Ano1-positive cells in the mucosa and sub-mucosa (see above, Fig 2F) were all negative for Kit (Fig. 4A) and had the morphology and location of myofibroblasts. In the muscularis propria, Kit-negative, Ano1-positive cells were not observed.

Mast cells were not positive for Ano1, although mast cells (Kit-positive, Ano1-negative) were detected in all layers of the gastrointestinal tract. The mucosa and submucosa contained the greatest numbers of mast cells (Fig. 4A), but mast cells were also observed in the muscle layers (Fig. 4B). The mast cells were distinguished by bright Kit- labeling with round cell bodies and absence of processes. No Kit-positive cells with mast cell morphology were observed to be immunoreactive for Ano1. This was confirmed by double labeling human colon with antibodies to mast cell tryptase and to Ano1. Tryptase-positive mast cells were frequently found in the mucosa and submucosa as well as in the muscularis propria (Fig. 4C). Ano1-immunoreactivity never
colocalized with mast cell tryptase (Fig. 4C) confirming that mast cells do not express Ano1.

A small number of strongly Ano1-positive cells with clear ICC-like morphology were weakly positive for Kit when compared with adjacent Kit-positive ICC (Fig. 5A). These cells were observed most frequently in the circular muscle layer of the gastric body but were also detected in small numbers in the septa of the circular muscle layer of the small intestine. In the stomach, in three tissues, a total of 416 Ano1-positive ICC were counted and 3.2±1.5% (1.57, 1.88 and 6.2%) of Ano1-positive ICC were weakly positive for Kit. All Kit-positive cells with ICC morphology were positive for Ano1.

Ano1-immunoreactivity in mouse gastrointestinal tract. The rabbit anti-Ano1 antiserum was used to detect ICC in mice. Co-localization of Ano1-immunoreactivity with Kit-immunoreactivity was examined in whole mount preparations of the muscularis propria from the fundus and body of the stomach, small intestine, and proximal and distal colon. As in human tissue, the pattern and distribution of Ano1-immunoreactivity was consistent with labeling of ICC and overlapped exactly with Kit-immunoreactivity (Figs. 6-8). As in human tissues, we detected no labeling of ICC-like cells by any of the secondary antibodies in the absence of primary antiserum. We did not obtain specific immunolabeling with the mouse antiserum to Ano1 on mouse tissues. In the small intestines, ICC were also labeled with the chicken antibody (Abcam), but the labeling was inconsistent and of poor quality.

In mouse stomachs, doubly labeled, bipolar ICC were observed in the muscle layers of the fundus (Fig. 6A), body (Fig. 6B), and antrum (Fig. 6C). Networks of
multipolar ICC-MY were labeled in the gastric body (Fig. 6D) and additionally a small number of ICC-SM in the gastric antrum were identified by double labeling (Fig. 6E). In the small intestine of mice, both ICC-DMP and ICC-MY were labeled clearly with both antisera (Fig. 7). The ICC-DMP were bipolar in shape (Fig. 7A) and the ICC-MY formed a dense network around the myenteric ganglia (Fig. 7B). ICC-IM and ICC-MY positive for Ano1 and Kit were also identified in the mouse proximal colon (Fig. 7C and D). Doubly positive ICC with stellate morphology were observed on the serosal surface of the longitudinal muscle (Fig. 7E). In addition, ICC with large cell bodies and 2 or more widely branching processes were also doubly labeled for Ano1 and Kit in the submucosal plexus. In the distal colon, ICC-SM (Fig. 8A) and ICC-IM (Fig. 8B and 8D) had more extensive processes than in the proximal colon but were also doubly labeled for Ano1 and Kit. Doubly labeled ICC-MY of the distal colon had larger cell bodies (Fig. 8C) than ICC-MY of other parts of the mouse gastrointestinal tract.

The brightness of the fluorescently labeled structures varied considerably for both Kit and Ano1-immunoreactive cells. No cells with ICC morphology were however detected that were Ano1-positive and completely Kit-negative or Kit-positive and completely Ano1-negative. No Kit-positive, Ano1-negative mast cells were detected.

**DISCUSSION**

This study identifies Ano1 as a new, selective molecular marker for all classes of ICC in the stomach, small intestine, and large intestine of humans and mice that permits the immunochemical identification of these cells independent of Kit.
The demonstration that Kit is a selective marker for ICC (23) permitted discoveries that explain basic mechanisms in the regulation of gastrointestinal motility in health and disease. In human disease, Kit has been used as a marker to follow loss of ICC and changes in network density associated with a variety of diseases (9). Use of neutralizing antibodies that inhibit signaling has demonstrated that Kit is required for normal development and maintenance of ICC networks (33). Kit function and regulation of ICC numbers and network density seem to be associated intimately. While Kit remains an excellent marker for ICC, an advantage of Ano1 is that it represents a novel marker that has no known link to Kit, and so use of Ano1 should permit examination of ICC independent of Kit. Also, the Ano1 antisera do not label mast cells (Kit and tryptase-positive cells), so there is no need to take into account mast cells when quantifying ICC using Ano1. Also, this increases the accuracy of ICC quantification when using Ano1 protein and/or mRNA as compared to Kit. Ano1 also had the additional benefit of identifying ICC-like cells that were positive for Ano1 but weakly positive for Kit. These “Kit-dim” ICC were only marginally brighter for Kit-immunofluorescence than the background fluorescence, and it is feasible that these cells would have been missed if labeled only for Kit. The only caveat is that in mouse but not human tissues, immunolabeling for Ano1 using the commercially available antibodies was not quite so clear as immunolabeling for Kit.

The identification of Ano1-positive cells as ICC was based on the co-expression of Ano1 and Kit-immunoreactivity as well as the ICC-like morphology of the doubly labeled cells. Also, the distribution of the Ano1-positive, Kit-positive cells in the gastrointestinal tract was exactly as expected for ICC in those tissues (as reviewed by...
These observations were confirmed by using two different antisera to label human tissues and were repeated in studies using one of the antisera on mouse gastrointestinal tract. The Ano1 antisera labeled intramuscular, myenteric, septal, submuscular, subserosal and DMP-ICC. Therefore Ano1 labels all classes of ICC.

Immunohistochemical evidence for Ano1 protein expression in ICC is supported by studies investigating differential expression of genes in ICC when compared to overall expression of those genes in the mouse gastrointestinal tract. Ano1 mRNA was 4-8 fold more highly expressed in myenteric and DMP-ICC compared to all cells in the mouse jejunum (6).

The authenticity of Ano1-immunoreactivity in our studies is confirmed by the use of antibodies raised in different species to different antigens derived from the sequence of Ano1 (8) and the lack of labeling in the colon from Ano1 knockout mice. A third antiserum raised in chickens was also tested. The signal from the chicken antiserum showed unacceptably high levels of background signal but signal from ICC-like cells represented the only signal that overlapped with the signals from the rabbit and/or mouse antisera. The mouse antiserum used to identify Ano1-positive cells in human tissues was raised against peptides with the primary amino acid sequences (8) of regions of the protein predicted to be on the intracellular surface of the plasma membrane (5). These Ano1-derived antigens had no sequence homology to the peptide sequence of Kit (8) so it is very unlikely that the antibodies were cross-reacting with Kit on ICC. Also, in situ hybridization for Ano1 (DOG1) RNA correlates well with the Ano1 immunohistochemistry (38) and the probe sequences have no overlap with Kit. In addition, the failure of Ano1 antisera to recognize strongly Kit-positive mast cells further
supports the conclusion that the antisera studied do not recognize Kit. Our data are in agreement with the assertion that Ano1 is not expressed on mast cells (8), and we have no evidence to support earlier suggestions that Ano1-immunoreactivity is present on mast cells (38).

It is possible that the antibodies were recognizing proteins containing peptide sequences similar to the Ano1-sequences. Therefore, we checked for similarities by BLAST search (1) and determined that the peptide sequences in the regions of the antigen were only similar to the sequences of analogous regions in other members of the Ano/TMEM16 family of proteins, and even then, the identity was less than 50%. Furthermore, mRNAs for members of the Ano/TMEM16 family of proteins that were not Ano1 were not over-represented in ICC compared to the surrounding tissue (6), so it is unlikely that the antisera were cross reacting with any protein related to Ano1.

Ano1-positive, Kit-negative cells in the mucosa and sub-mucosa were not in regions where ICC are detected, and unlike the weakly Kit-positive cells did not resemble either mast cells or ICC. It is not clear what those cells might be, but other investigators have identified Ano1 in many other tissues (18), specifically in the epithelia of foregut and airways (28) and mammary and salivary glands (30, 41). The morphologies of the Ano1-positive, Kit-negative cells at the base of the crypts of the mucosa were similar to those of myofibroblasts, but confirmation of their identity was not obtained in this study. The distribution of Ano1-immunoreactivity in the submucosal and mucosal regions of the mouse gastrointestinal tract was not investigated in the present study.

The function of Ano1 in ICC is not known. The labeling pattern we observed in both mouse and human ICC is consistent with the recent identification of Ano1 as a
membrane-associated, $\text{Ca}^{2+}$-activated $\text{Cl}^{-}$ conductance (5, 30, 41). What function Ano1 plays in ICC physiology and development remains to be determined. Kit plays a critical role in the development and maintenance of ICC (4, 9, 19, 21, 23, 33, 36) so changes in its expression may temporally precede changes in ICC mass. If Ano1 did not have a similar developmental role, it could perhaps be utilized as a more accurate indicator of ICC numbers, especially in pathologies involving changes in Kit signaling (17).

In conclusion, Ano1 is expressed on Kit-positive ICC in the human and mouse gastrointestinal tract. Ano1 represents a new highly selective molecular marker for studying the distribution and fate of ICC.
Acknowledgments

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Disclosures

The authors report no conflict of interest.
References


35. **Vanderwinden JM, Rumessen JJ, de Kerchove d'Exaerde A, Jr., Gillard K, Panthier JJ, de Laet MH, and Schiffmann SN.** Kit-negative fibroblast-like cells


Figure Legends

Fig. 1. Ano1-immunoreactivity in human gastrointestinal tract. (A) Labeling patterns for Ano1-immunoreactivity using the mouse anti-Ano1 from Applied Genomics (left) and the rabbit anti-Ano1 from Abcam (middle) in circular muscle of human stomach (43yr F). The merged image shows complete colocalization between mouse (red) and rabbit (green) signals. Nuclei were counter-stained with DAPI (blue). (B) Ano1-immunoreactivity across full thickness of human stomach tissue (33yr F). (C) Ano1-immunoreactivity across full thickness of jejunal tissue (41yr M). Arrowhead indicates ICC in deep muscular plexus. (D) Ano1-immunoreactivity in human colon (40yr F). Arrowheads indicate ICC-SM. Panel A is a single image collected at 400X magnification. Panels B, C and D are montages of images collected at 200X magnification.

Fig. 2. High resolution images of Ano1-immunoreactivity in all subclasses of human ICC. (A) Ano1-positive ICC-IM in circular muscle of the colon (36yr F). (B) Positively labeled septal ICC in circular muscle of the jejunum (28yr F). (C) ICC-MY in colon (36yr F). (D) ICC-DMP in jejunum (28yr F). (E) ICC-SM in colon (40yr F) (F) Left: Ano1-positive cells that did not have ICC-like morphology in mucosa from colon (40yr F). Right: Image of the DAPI-labeled nuclei in the same field shown on the left. Dotted line marks nuclei of epithelial cells in a crypt for reference. Scale bar = 20 μm for all panels. All images were collected at 400X magnification.

Fig. 3. Co-localization of Ano1 and Kit-immunoreactivity in the human gastrointestinal tract. In all merged images, the signal obtained with rabbit antisera is shown in red and
the signal from mouse antisera is shown in green. Blue color shows DAPI staining in nuclei. (A) Immunoreactivity for Ano1 and Kit were coincident when using mouse anti-Ano1 from Applied genomics (left) and rabbit anti-Kit from IBL (middle) to label human colonic ICC (40yr F). (B) Immunoreactivity for Ano1 and Kit were also coincident when using rabbit anti-Ano1 from Abcam (left) and mouse anti-Kit from Labvision (middle) to label human colonic ICC (53yr M). (C) Colocalization of Ano1 and Kit-immunoreactivity in ICC-IM in the circular muscle from colon (40yr F). (D) Colocalization of Ano1 (left) and Kit-immunoreactivity (middle) in myenteric and septal ICC of human jejunum (41yr M). (E) Ano1 (left) and Kit-immunoreactivity (middle) in longitudinal muscle of colon (36yr F). (F) Single, confocal slice collected at 600X magnification in circular muscle of stomach (25yr F). Ano1 (left) and Kit-immunoreactivity (middle) are shown.

Fig. 4. Ano1 labels Kit-negative cells in the mucosa but does not label Kit-positive mast cells. (A) In colonic mucosa Ano1 (left, green) and Kit-immunoreactivity (middle, red) does not colocalize as shown in the merged image (right, DAPI stained nuclei in blue). This image is from the same field as shown in Fig 2F. (B) In the muscularis propria, Kit-positive cells (arrowheads, middle, red) with mast cell morphology do not express Ano1-immunoreactivity (left, green). Tissue was from stomach (34yr F). (C) Mast cell lack of immunoreactivity to Ano1 was confirmed by double labeling human colon sections with antibodies to mast cell tryptase (arrowheads, middle, red) and Ano1 (left, green) confirming that mast cells do not express Ano1.
Fig. 5. Presence of weakly Kit-positive (Kit-dim) ICC in the human stomach. (A) Volume rendered images from confocal stacks collected at 600X magnification showing Ano1-immunolabeling (left, red) and Kit-immunolabeling (middle, green) illustrating presence of weakly Kit-positive but Ano1-bright cells with ICC morphology in circular muscle layer of the stomach (25yr F). Arrowhead points to one of these weakly Kit-positive/Ano1-positive ICC.

Fig. 6. Ano1 antisera label ICC in adult murine stomach. Representative projection images of confocal stacks collected at 400X magnification from a whole mount incubated with rabbit anti-Ano1 antiserum (Abcam, left, green) and ACK2, a rat anti-Kit antibody (middle, red). The merged images are on the right. ICC-IM are shown from (A) fundus, (B) corpus and (C) antrum. (D) Myenteric region ICC in corpus and (E) Sub-mucosal ICC in antral region.

Fig. 7. Ano1 antisera label ICC in adult murine jejunum and proximal colon. Representative en face projection images of confocal stacks collected at 400X magnification from a whole mount incubated with rabbit anti-Ano1 antiserum (Abcam, green) and ACK2, a rat anti-Kit antibody (red). (A) Deep muscular plexus ICC are shown from jejunum. Arrowhead marks an Ano1-positive, Kit-dim ICC. (B) Myenteric region ICC from jejunum. Asterisk marks a group of Ano1-positive, Kit-dim ICC. (C) ICC-IM from circular muscle of proximal colon, (D) myenteric region ICC in proximal colon and (E) subserosal ICC in proximal colon.
Fig. 8. Ano1 antisera label ICC in adult murine distal colon. Representative en face projection images of confocal stacks collected at 400X magnification from a whole mount incubated with rabbit anti-Ano1 antiserum (Abcam, green) and ACK2, a rat anti-Kit antibody (red). Merged images are on the right. (A) Sub-mucosal ICC, (B) ICC-IM from circular muscle layer, (C) myenteric region ICC and (D) ICC-IM in longitudinal muscle.
Table 1. Patient details for tissues studied.

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<td>Stomach 3</td>
<td>34</td>
<td>Female</td>
<td>Body</td>
</tr>
<tr>
<td>Small intestine 1</td>
<td>28</td>
<td>Female</td>
<td>Jejunum</td>
</tr>
<tr>
<td>Small intestine 2</td>
<td>34</td>
<td>Female</td>
<td>Jejunum</td>
</tr>
<tr>
<td>Small intestine 3</td>
<td>41</td>
<td>Male</td>
<td>Jejunum</td>
</tr>
<tr>
<td>Colon 1</td>
<td>36</td>
<td>Female</td>
<td>Sigmoid</td>
</tr>
<tr>
<td>Colon 2</td>
<td>40</td>
<td>Female</td>
<td>Ascending</td>
</tr>
<tr>
<td>Colon 3</td>
<td>53</td>
<td>Male</td>
<td>Ascending</td>
</tr>
</tbody>
</table>
Table 2. Antisera used in this study.

<table>
<thead>
<tr>
<th>Cat #/Clone</th>
<th>Target</th>
<th>Host Species</th>
<th>Supplier</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0732</td>
<td>DOG-1</td>
<td>Mouse</td>
<td>Applied Genomics</td>
<td>1:400</td>
</tr>
<tr>
<td>Ab53212</td>
<td>TMEM16A</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>0.25 μg/ml (Human)</td>
</tr>
<tr>
<td></td>
<td>(DOG-1)</td>
<td></td>
<td></td>
<td>2 μg/ml (mouse)</td>
</tr>
<tr>
<td>Ab16293</td>
<td>TMEM16A</td>
<td>Chicken</td>
<td>Abcam</td>
<td>2 μg/ml</td>
</tr>
<tr>
<td></td>
<td>(DOG-1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DOG-1.1</td>
<td>Mouse</td>
<td>House</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>18101</td>
<td>Kit</td>
<td>Rabbit</td>
<td>IBL</td>
<td>0.20 μg/ml</td>
</tr>
<tr>
<td>Ms-483-P0</td>
<td>Kit</td>
<td>Mouse</td>
<td>Lab Vision</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td></td>
<td>(monoclonal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACK 2</td>
<td>Kit</td>
<td>Rat</td>
<td>House</td>
<td>5 μg/ml</td>
</tr>
<tr>
<td></td>
<td>(monoclonal)</td>
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<td></td>
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<tr>
<td>Sc-32889</td>
<td>Mast cell tryptase</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>Donkey-Cy5</td>
<td>Mouse IgG</td>
<td>Donkey</td>
<td>Chemicon</td>
<td>7.5 μg/ml</td>
</tr>
<tr>
<td>Donkey-Cy3</td>
<td>Mouse IgG</td>
<td>Donkey</td>
<td>Jackson</td>
<td>3.5 μg/ml</td>
</tr>
<tr>
<td>Donkey-Cy3</td>
<td>Rabbit IgG</td>
<td>Donkey</td>
<td>Chemicon</td>
<td>1.25 μg/ml</td>
</tr>
<tr>
<td>Donkey-Cy5</td>
<td>Rabbit IgG</td>
<td>Donkey</td>
<td>Chemicon</td>
<td>7.5 μg/ml</td>
</tr>
<tr>
<td>AF488</td>
<td>Rabbit IgG</td>
<td>Chicken</td>
<td>Invitrogen</td>
<td>10 μg/ml</td>
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<tr>
<td>AF488</td>
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<td>Invitrogen</td>
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<td>Goat</td>
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<td>10 μg/ml</td>
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</tbody>
</table>
A) Human Stomach (Circular Muscle)

Rb anti-Ano1

Ms anti-Kit

Merged

50 μm
A) Jejunum (Deep Muscular Plexus)

B) Jejunum (Myenteric Plexus)

C) Proximal Colon (Circular Muscle)

D) Proximal Colon (Myenteric Region)

E) Proximal Colon (Longitudinal Muscle)