

Localized enteric neural deficiency and reduced nerve-mediated anorectal contractility in immunodeficient *Ret^{S812F/+}* mice

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Supplementary table 1. PCR primers set for genotyping.

Aim for screening	Sequence of primer set
<i>Rag2</i> target alleles	RAG2-F: 5'-GATTCCTGCTACCTCCACCT-3' RAG2-R4: 5'-TCACTCAAATCCTCTCTCTCCAGAGCATCTC-3' IL2R γ -PII: 5'-CCTCCGTGCAATCCATCTTGTTCAAT-3'
<i>Il2rg</i> target alleles	IL2R γ -P1I: 5'-CTGCTCAGAATGCCTCCAATTCC-3' IL2R γ -PII: 5'-CCTCCGTGCAATCCATCTTGTTCAAT-3' IL2R γ -PIII: 5'-GATCCAGATTGCCAA GGTGAGTAG -3'
<i>Sirpa</i> mutations	Sirpa-common: 5'-AAATCAGTGTGTGTTGCTGCT-3' Sirpa-B6: 5'-TGTGTCAGGCTCTGA-3' Sirpa-NOD: 5'-TATTTCTGTGTCAGGCCGA-3'
<i>Ret</i> mutations	Ret-F: 5'-ATAGATCCAGAGCAGGTCTGTGCC-3' Ret-R: 5'-TGGCAGCTAAGTCCCGATGTACAA-3'

Rag2, recombination activating gene 2; *Il2rg*, interleukin-2 receptor gamma; *Sirpa*, signal regulatory protein alpha; *Ret*, rearranged during transfection; P, probe; F, forward primer; R, reverse primer.

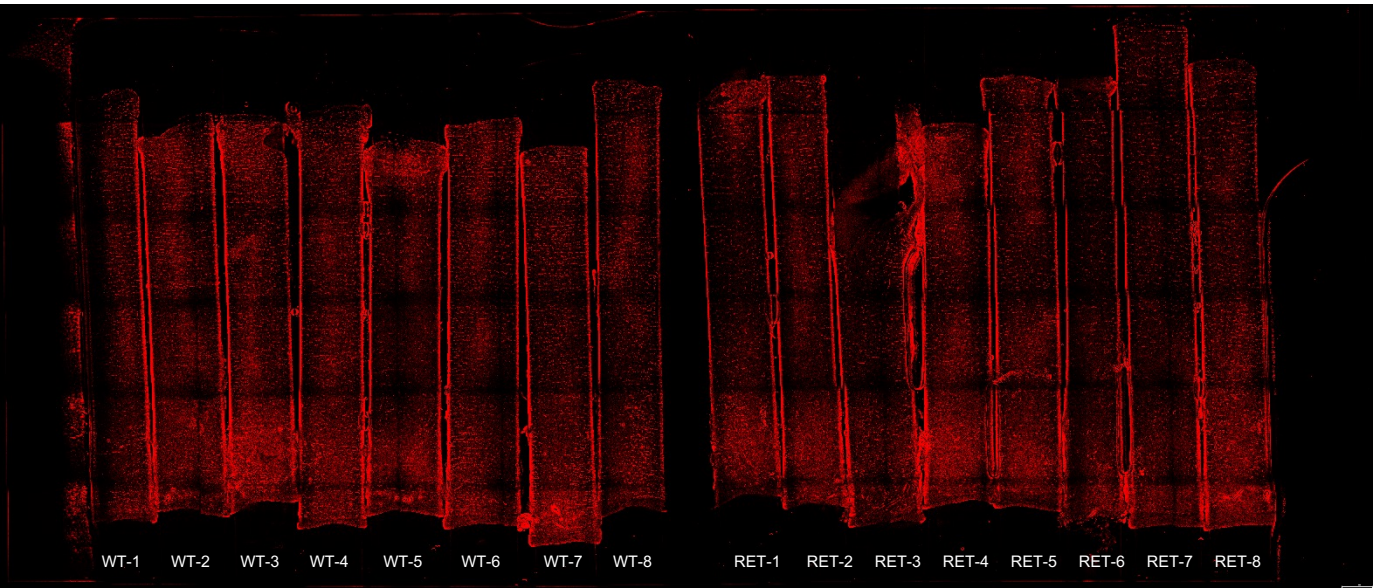
Supplementary table 2. Sequences of primers for RT-qPCR.

Gene	Transcript ID	Sequence or Assay ID
<i>Actb</i>	NM_007393.5	P: CACAGCTTCTTTGCAGCTCCTTCGTTGCC F: 5'-CAACGCGCAGCCACTGTC R: 5'-GTCATCCATGGCGAACTGGTG
<i>Tubb3</i>	NM_023279.2	P: AGGTTCCAAGTCCACCAGAATGGCCCTG F: 5'-TGAGGCCTCCTCTCACAAGTAT R: 5'-AGCACCACTCTGACCAAAGATAAAG
<i>Chat</i>	NM_009891.2	P: AGGGACTTGTCATACCAACGATTCGCTCCA F: 5'-GGATGGTCCAGGCACTGGA R: 5'-CTCGGCCCAACCACAAACTG
<i>Phox2b</i>	NM_008888.3	P: CAGAACCGCCGGGCTAAGTTTCGCAA F: 5'-GGCGAGAGTCCAGGTGTGG R: 5'-CCGGGAGGAGTCAGACTTCTTC
<i>Nos1</i>	NM_008712.3	P: CAGTTTGCCGTCGAGGTCTCTGTCCAC F: 5'-AGCAGAGATGAAAGACACAGGAA R: 5'-GGGTTGTTGAGGACCACAGG
<i>Vip</i>	-	Mm00660234
<i>Calb2</i>	-	Mm00801461

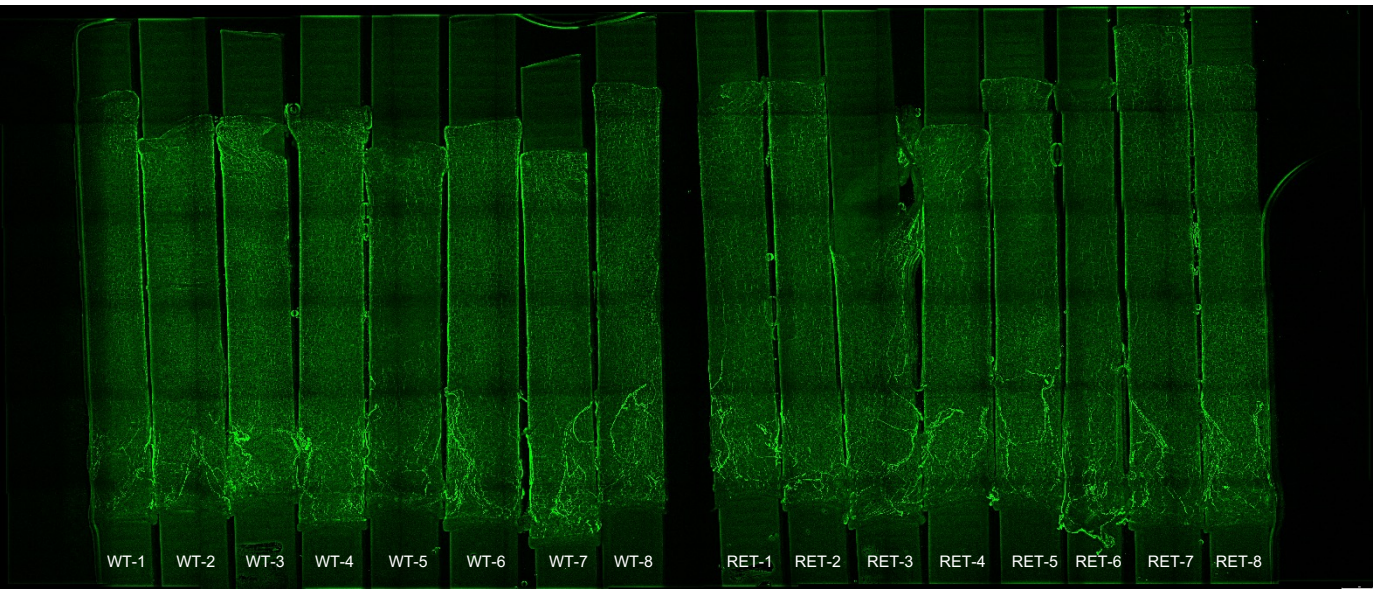
Actb, actin beta; *Tubb3*, tubulin beta-3 chain; *Chat*, choline acetyltransferase; *Phox2b*, paired-like homeobox 2b; *Nos1*, nitric oxide synthase 1; *Vip*, vasoactive intestinal polypeptide; *Calb2*, calbindin2; P, probe; F, forward primer; R, reverse primer.

Supplementary figure 1.

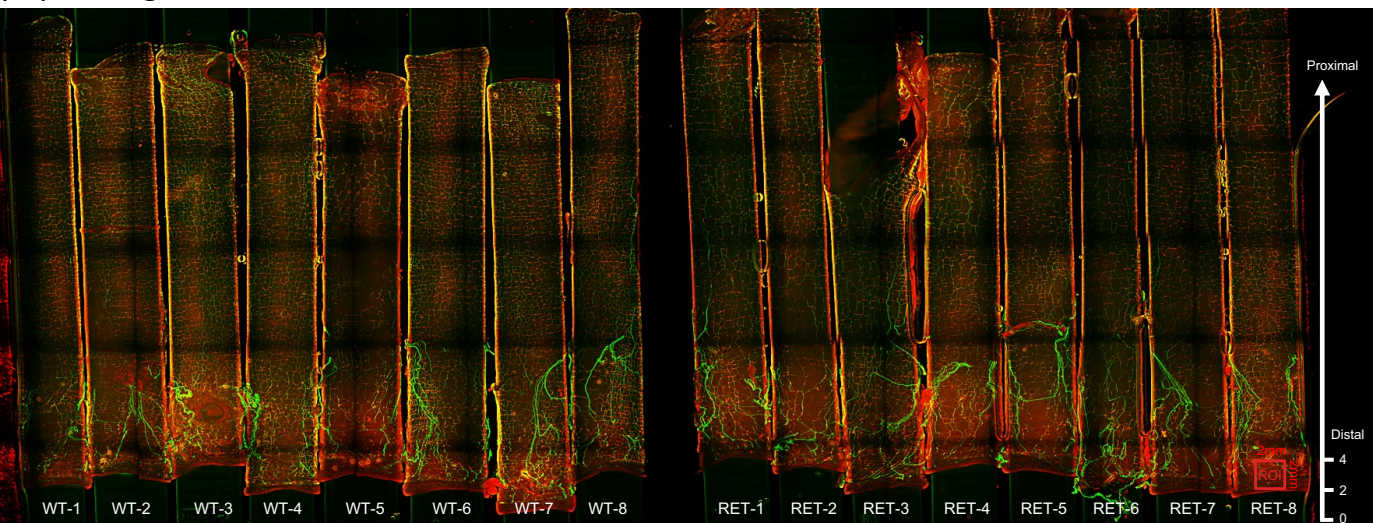
(A) HuC/D



(B) Tubb3

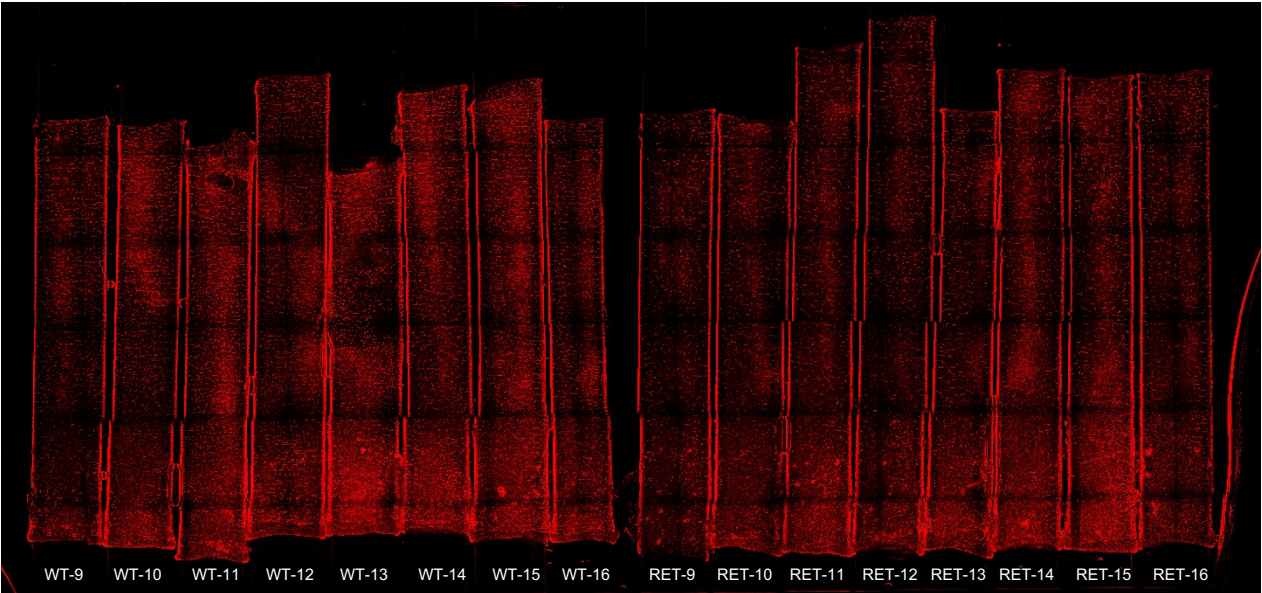


(C) Merge

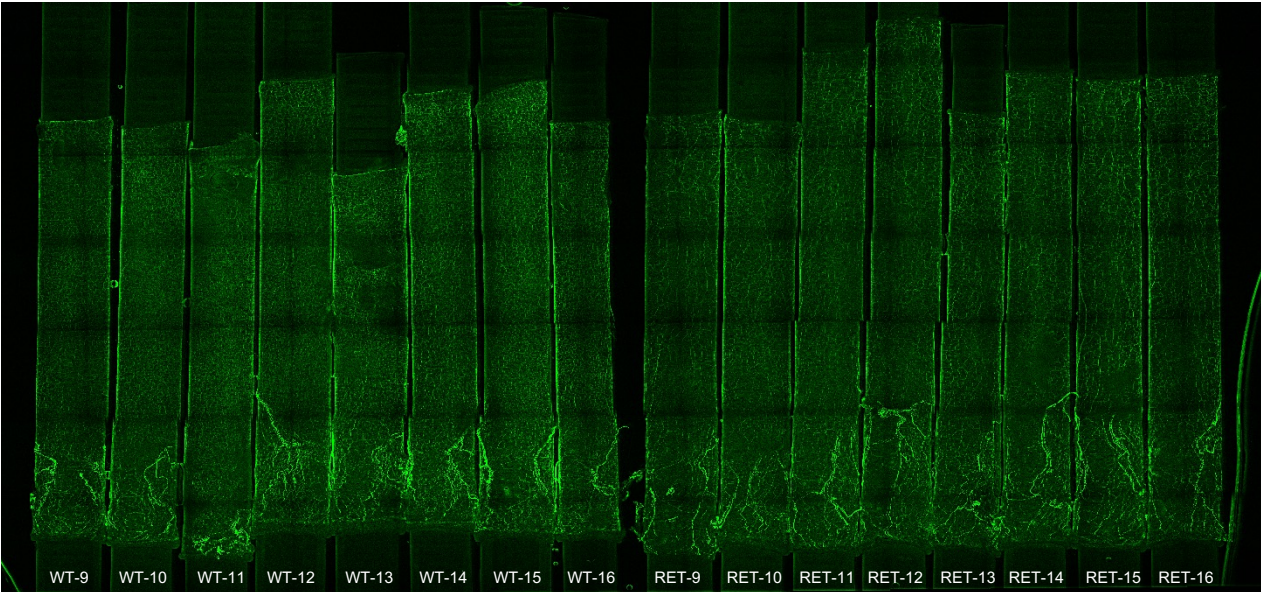


Supplementary figure 1.

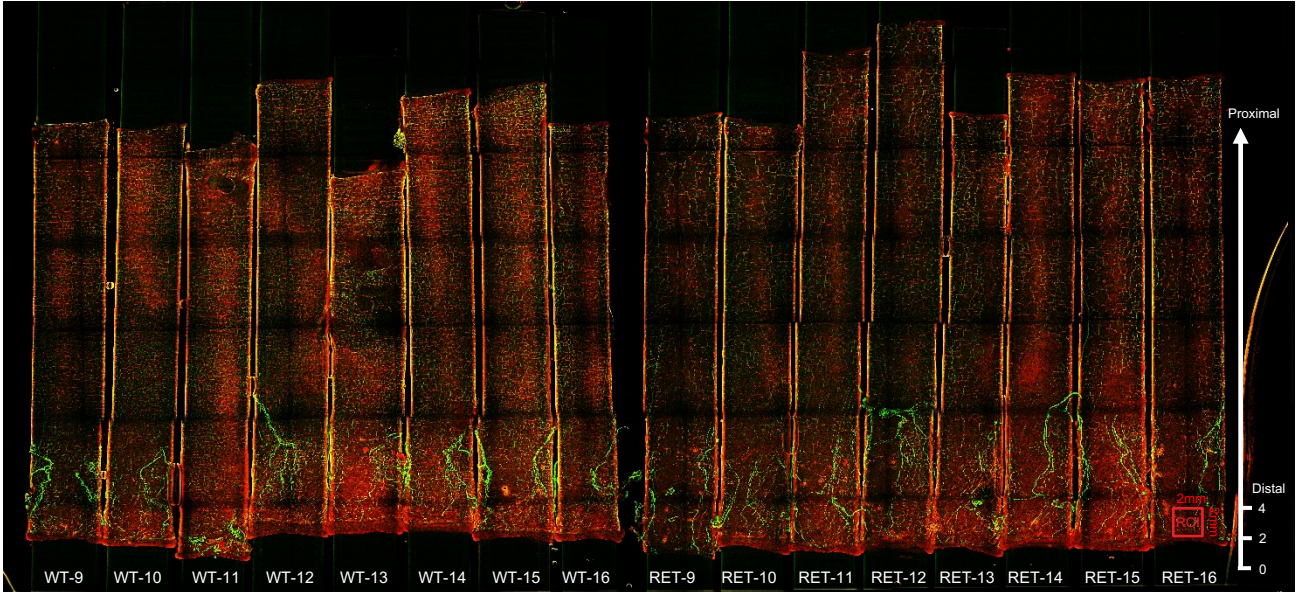
(D) HuC/D



(E) Tubb3



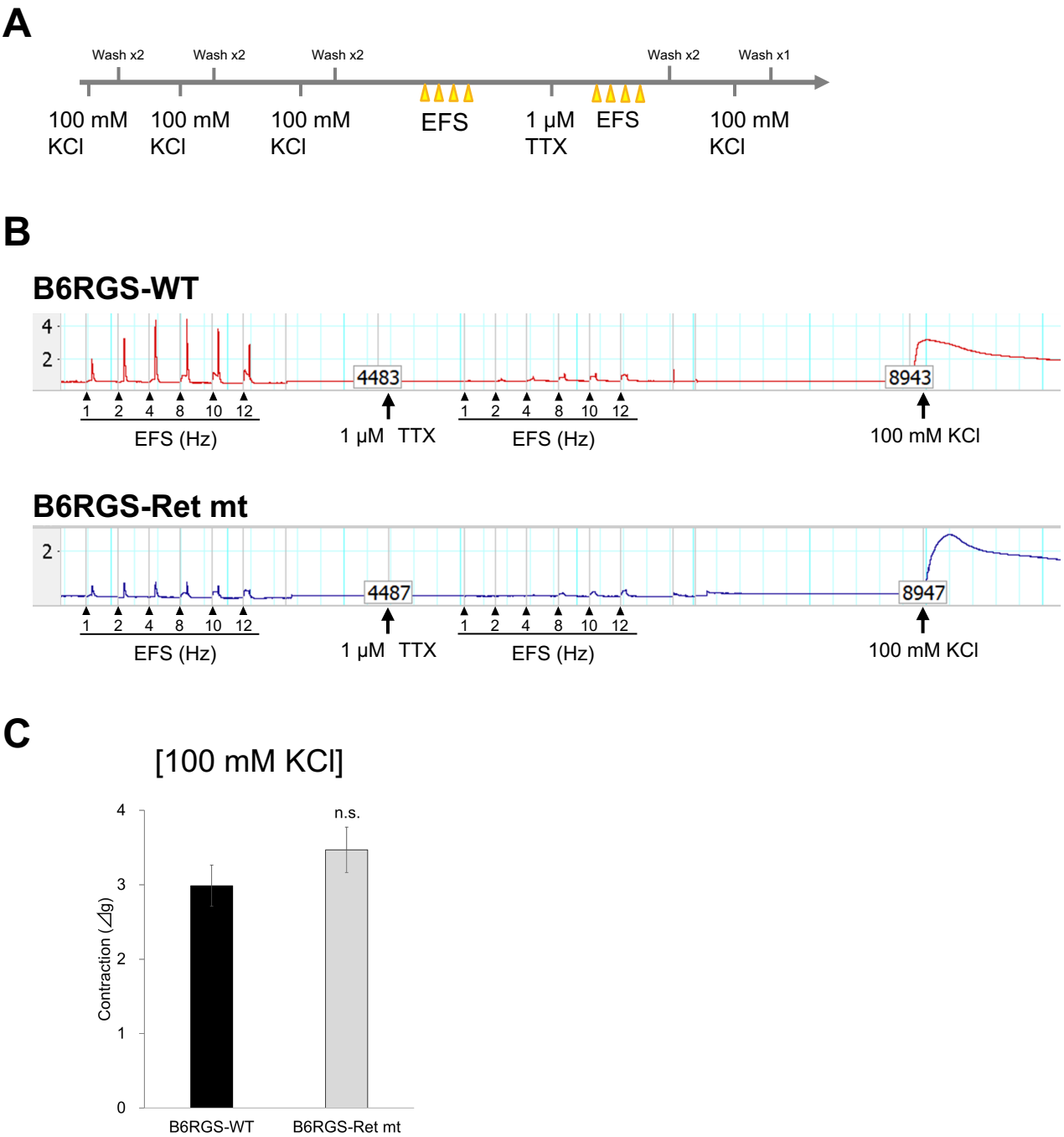
(F) Merge



Supplementary figure 1. Whole-mount colon immunostaining.

Immunohistochemical staining of the whole colon with HuC/D (A, D), Tubb3 (B, E) and merge (C, F) in B6RGS-wild type (B6RGS-WT) and B6RGS-*Ret*^{S812F/+} mouse (B6RGS-Ret mt) are shown. After removal of the anorectal segment for the organ bath assay, the remaining colon was rinsed with sterile phosphate-buffered saline (PBS) to remove stool. A small plastic cable tie was inserted from the distal end to maintain luminal patency, and the tissue was fixed in 1% neutral-buffered formaldehyde overnight at 25°C. For quantitative analysis of each specimen, a 2 mm x 2 mm region of interest (ROI, red square) was defined at a consistent distance from the anal verge to capture the most distal phenotype shown in (C) and (F). Sample WT-1 to 16 from B6RGS-WT; Sample RET-1 to 16 from B6RGS-Ret mt.

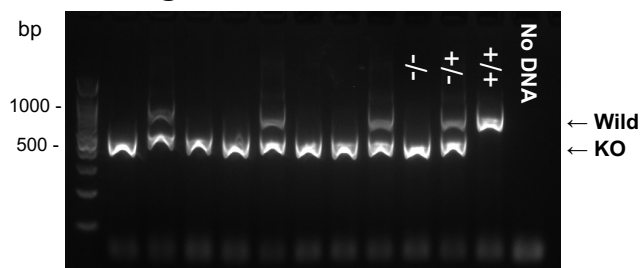
Supplementary figure 2.



Supplementary figure 2. Contractile response of anorectal tissue in organ bath assay. (A) Experimental flow of organ bath assay. Tetrodotoxin (1 μ M) was applied 5 min before EFS stimulation to confirm that EFS-evoked contractions were nerve dependent. (B) Representative entire raw chart of anorectal contraction. (C) Comparison of 100 mM KCl-induced muscle related-contraction between B6RGS-wild type (B6RGS-WT) and B6RGS-*Ret*^{S812F/+} mouse (B6RGS-Ret mt). The data is shown in the results of the final contractile response by 100 mM KCl following EFS stimulation. Data are presented as mean $\pm$ SEM by 16 mice per group. n.s., not significant (Student's *t* test); TTX, tetrodotoxin; EFS, electric field stimulation

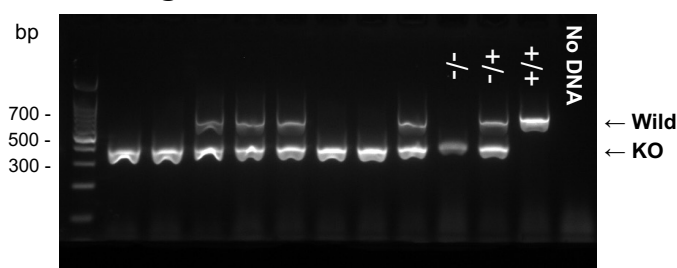
Supplementary figure 3.

A *Rag2* KO



✖Figure 1C

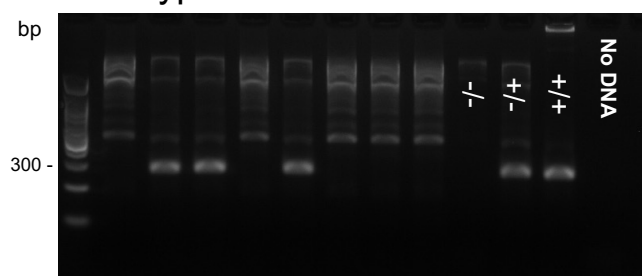
B *Il2rg* KO



✖Figure 1D

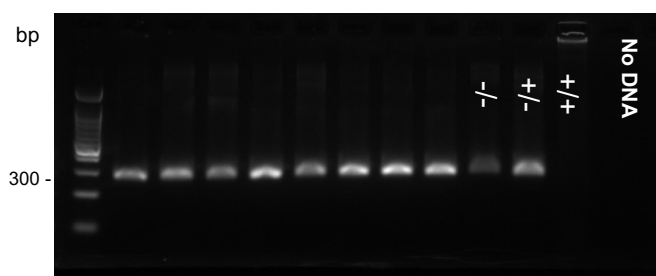
C *Sirpa* 6bp deletion

Wild type PCR



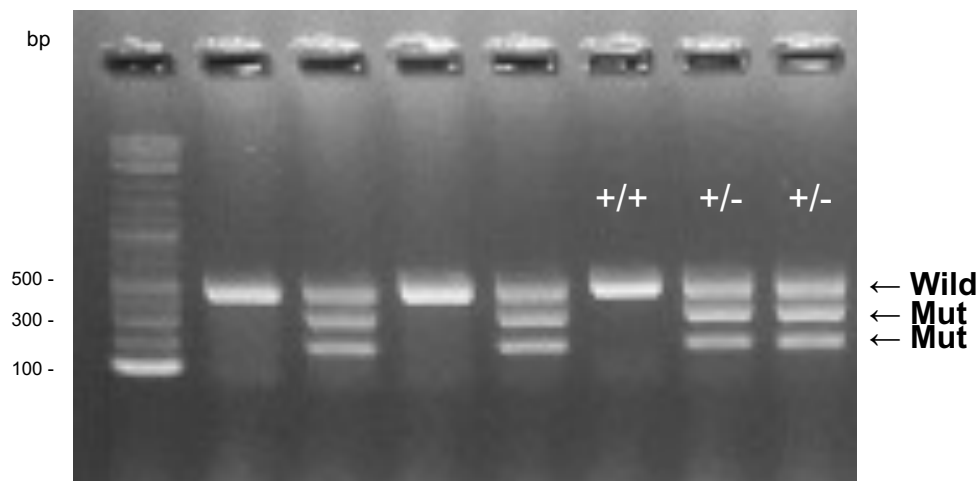
✖Figure 1E (left)

Deletion PCR



✖Figure 1E (right)

D *Ret* mutation



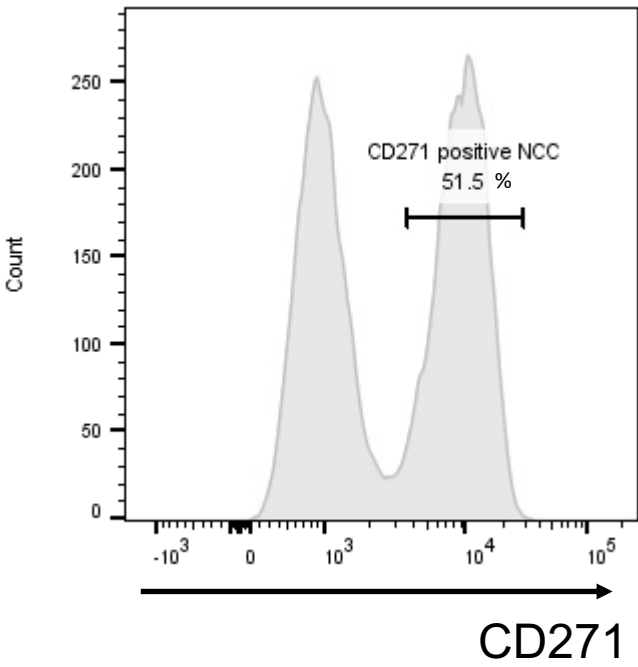
✖Figure 1F

Supplementary figure 3. Representative electropherogram in genotyping.

The PCR products for *Rag2* KO (A), *Il2rg* KO (B), *Sirpa* 6bp deletion (C), and *Ret* mutation (D) were analyzed by agarose gel electrophoresis. Expected PCR products are shown as follows; *Rag2* WT, 878 bp; *Rag2* KO, 520 bp; *Il2rg* WT, 660 bp; *Il2rg* KO, 340 bp; *Sirpa* WT (Left), 279 bp; *Sirpa* deletion (Right), 279 bp. The PCR product from *Ret* mutations was digested with the *MspI* restriction enzyme at 37° C for 1 h. The wild-type allele did not contain the *MspI* site, resulting in a 450-bp band, whereas the *Ret* S812F mutant allele resulted in 300-bp and 150-bp bands.

WT, wild type; KO, knockout; NOD, non-obese diabetic; PCR, polymerase chain reaction; bp, base pair; *Rag2*, recombination activating gene 2; *Il2rg*, IL-2 receptor gamma; *Sirpa*, signal-regulatory protein alpha

Supplementary figure 4.



Supplementary figure 4. Characterization of iPSC-vNCCs.

Expression of neural crest cell surface marker in vagal neural crest cells from human pluripotent stem cell (iPSC-vNCC). Gray histogram represent FACS labeling with anti-CD271 antibody.