Supplemental Methods

Mice

ICR and BDF1 were purchased form SLC (Hamamatsu, Japan). Male nude mice of ICR background were purchased from Charles River Japan, Inc. GFP transgenic mouse line (ICR background) was previously established in our laboratory using pCAG-EGFP as a transgene via ICSI mediated transgenesis [1].

Production of phox2b del5 and del8 mutant mice from chimeric mice

As we failed to obtain sufficient numbers of mutant embryos from phox2b del5- and del8- chimeric mice for developmental studies by standard methods, we employed an advanced method in which microinsemination is combined with transplantation techniques [2-4]. Briefly, chimeric mice for phox2b del5- and del8- mutant ES cells were produced by injecting the ES cells to the blastocysts of C57BL/6 or ICR carrying GFP transgene. The seminiferous tubules without green fluorescence, which presumed as ES cell derived tissue, were dissected from the testes of del5 or del8↔GFP transgenic chimeric mice under fluorescent microscopy [2]. Sperm prepared from the collected seminiferous tubules were injected into the oocytes of BDF1 background according to previous procedure [3, see below]. The resulting 2-cell stage embryos were transferred into pseudopregnant females, which were then analyzed at appropriate developmental periods.

As the progeny with mutant allele died soon after birth, the testes of newborn mice were rescued by transplanting them into the testes of nude mice [4]. Briefly, nude mice of ICR background at 4 wks of age were used as recipients for testicular tissue transplantation. To eliminate endogenous germ cells, busulfan was injected once (i.p.) at a dose of 40mg/kg. To decrease mortality, bone marrow (BM) cells prepared from non-treated nude mice were transplanted from tail vein after 3-5 days of busulfan treatment. Donor BM cells prepared from one non-treated nude mouse were suspended in 200 μl of PBS and 100 μl was transplanted into a buslfan-treated nude mouse. The nude mice were used as recipients 2-4 wks after BM transplantation. Donor testicular tissue was prepared from newborn heterozygous males, which died soon after birth. The testis was dissected and the tunica was removed with fine forceps. Testicular tissue was then cut into 1/4 with fine scissors in PBS and used as donor tissue. Two testicular pieces were transplanted into a recipient testis from the small incision of tunica. The testicular sperm were retrieved from the recipient testes at 8-10 wks after transplantation.

Intracytoplasmic sperm injection (ICSI) was performed according to a previous report [3] with minor modification. Briefly, the testicular cell suspension was prepared from the seminiferous tubules of grafts after transplantation or chimeric mouse testes by mincing them with fine scissors followed by gently agitating with micropipette. The testicular cell suspension was kept at 4^{o}C until ICSI. In the case of chimeras, the seminiferous tubule fragments without green fluorescence (i.e., donor ES derived spermatogenesis; [2]) were selectively collected under fluorescent microscope.

For oocyte preparation, superovulation was induced in BDF1 or C57BL/6 cre recombinase transgenic females (β-actin Cre) by an injection of 5 IU eCG, followed by a second injection of 5 IU hCG 48 h later. At 14 h post-hCG injection, the cumulus–oocyte complexes (COCs) were collected from the oviducts. Oocytes were freed from the cumulus cells by adding 0.1% bovine testicular hyaluronidase (ICN Biochemicals, Costa Mesa, CA) to the COC-containing medium. After the cumulus cells had dissociated, the oocytes were rinsed twice with Chatot, Ziomet, and Bavister (CZB) medium [5]. Approximately 2 μl of the sperm suspension was mixed with a drop of Hepes-Human Tubal Fluid (HTF) medium containing 10% (w/v) polyvinylpyrrolidone (PVP; IrvineScientific, Santa Ana, CA). The sperm head was separated from the tail by applying several piezo pulses to the neck region of the spermatid and the head was then injected into the oocyte according to the method described by Kimura and Yanagimachi (1995) [3]. The testicular cell suspension was replaced every 30 min during the ICSI experiment. The oocytes that survived ICSI were incubated in CZB medium at 37°C under an atmosphere of 5% CO_2. When the embryos reached the 2-cell stage, they were transferred to the oviducts of 0.5-dpc pseudopregnant ICR females.

Supplemental References

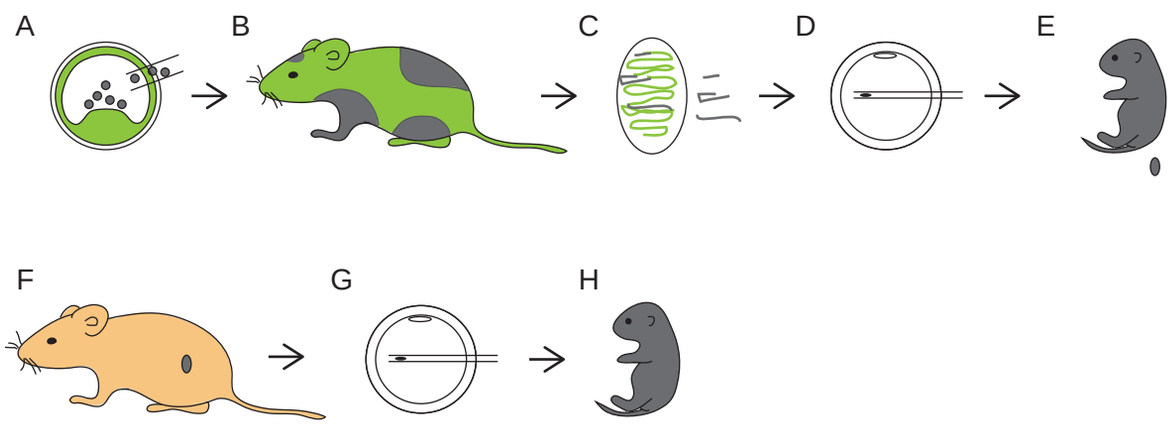
1. Perry AC, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y, Yanagimachi R. Mammalian transgenesis by intracytoplasmic sperm injection. Science. 1999; 14; 284:1180-1183.

2. Mizutani E, Ohta H, Kishigami S, Van Thuan N, Hikichi T, Wakayama S, Sato E, Wakayama T. Generation of progeny from embryonic stem cells by microinsemination of male germ cells from chimeric mice. Genesis. 2005; 43: 34-42.

3. Kimura Y, Yanagimachi R. Intracytoplasmic sperm injection in the mouse. Biol Reprod 1995; 52: 709–720.

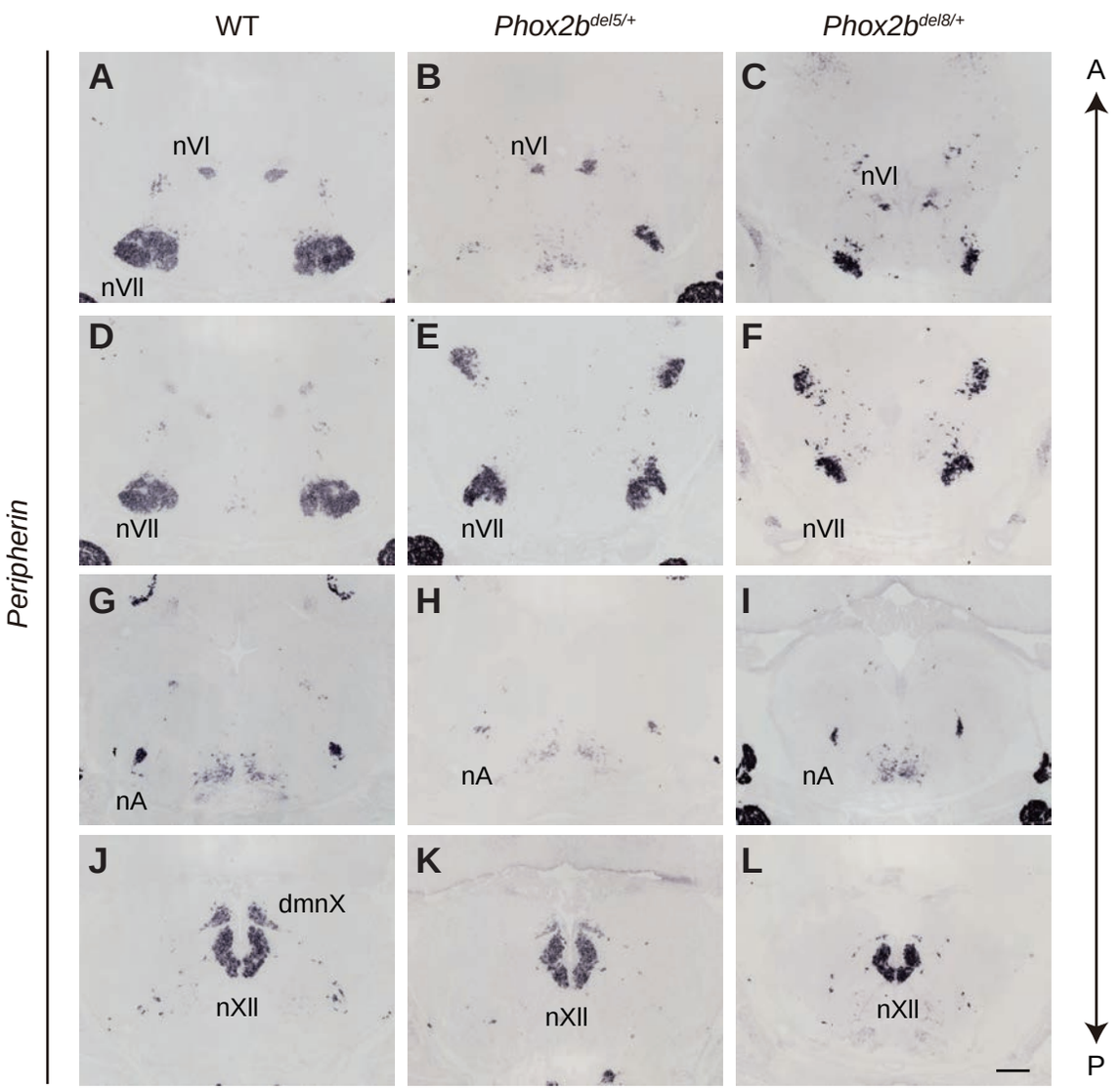
4. Ohta H and Wakayama T. Generation of normal progeny by intracytoplasmic sperm injection following grafting of testicular tissue from cloned mice that died postnatally. Biol Reprod. 2005; 73: 390-395.

5. Chatot CL, Lewis JL, Torres I, Ziomek CA. Development of 1-cell embryos from different strains of mice in CZB medium. Biol Reprod 1990; 42: 432–440.



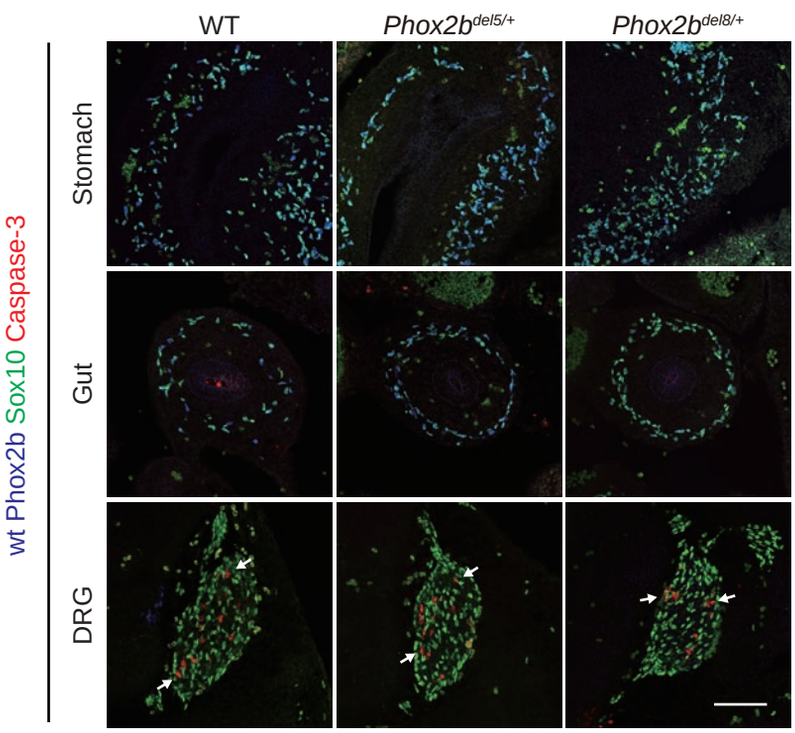
Supplemental Figure 1. Schematics showing propagation of NPARM *PHOX2B* mutant embryos

A procedure to generate del8 mutant embryos is shown. (A-B) Chimeric mice were obtained by injecting homologously recombined ES clones into blastocysts of mice engineered to express GFP ubiquitously. GFP-negative sperms were selected from the testes of the chimeras (C) and injected into oocytes of C57BL/6 mice (D), which resulted in generating a few newborn mice heterozygous for NPARM *PHOX2B* which died soon after birth (E). Testes of the male heterozygous mice (E) were xenografted into nude mice for ten weeks (F) to collect mature sperm, used for mass production of NPARM *PHOX2B* embryos (for more details, see Supplemental Materials and Methods).



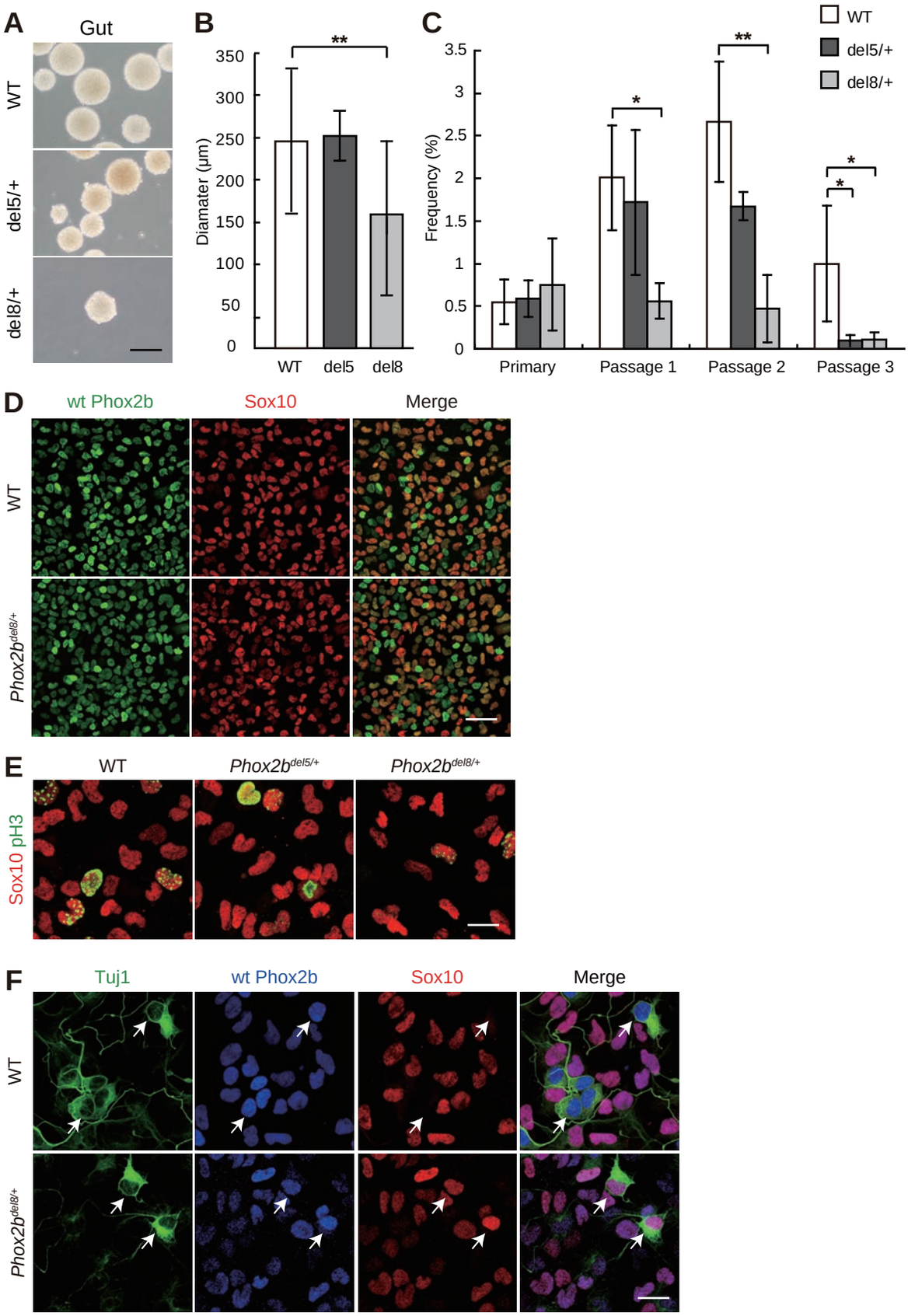
Supplemental Figure 2. Development of Phox2b-dependent hindbrain nuclei in NPARM *PHOX2B* embryos

Panels show results obtained by in situ hybridization analysis of the transverse sections of the hindbrain (E16.5) using riboprobes for peripherin, a type III intermediate filament protein expressed in neuronal cell populations which project their neurites to the peripheral targets (61). Individual horizontal panels represent sections at nearly identical anterior-posterior axial levels. Results are shown in an anterior-to-posterior order from top to bottom panels. A↔P; anterior ↔ posterior, nVI; the abducens nucleus, nVII; facial motor nucleus, nA; the ambiguus nucleus, dmnX; dorsal motor nucleus of the vagus, nXII; hypoglossal nucleus. Scale bar: 100 μm.

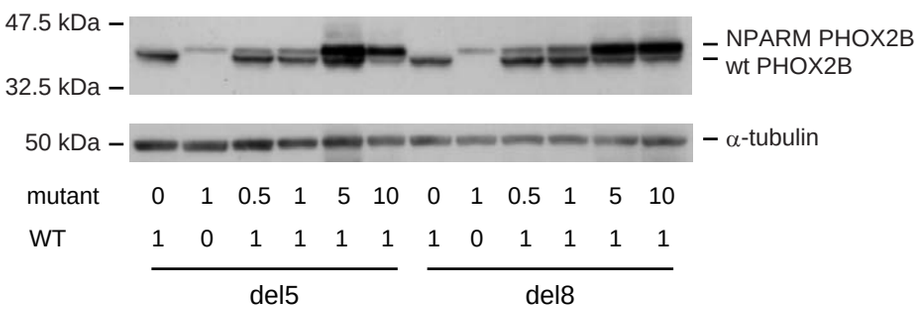


Supplemental Figure 3. Analysis of cell death of E12 embryos with activated caspase-3 antibody

Although caspase-3 positive cells are abundant in DRGs (bottom panels), no signals were detected in the stomach (top panels) and small intestine (middle panels) of both wt and NAPRM *PHOX2B* embryos. Scale bar: 100 μm.

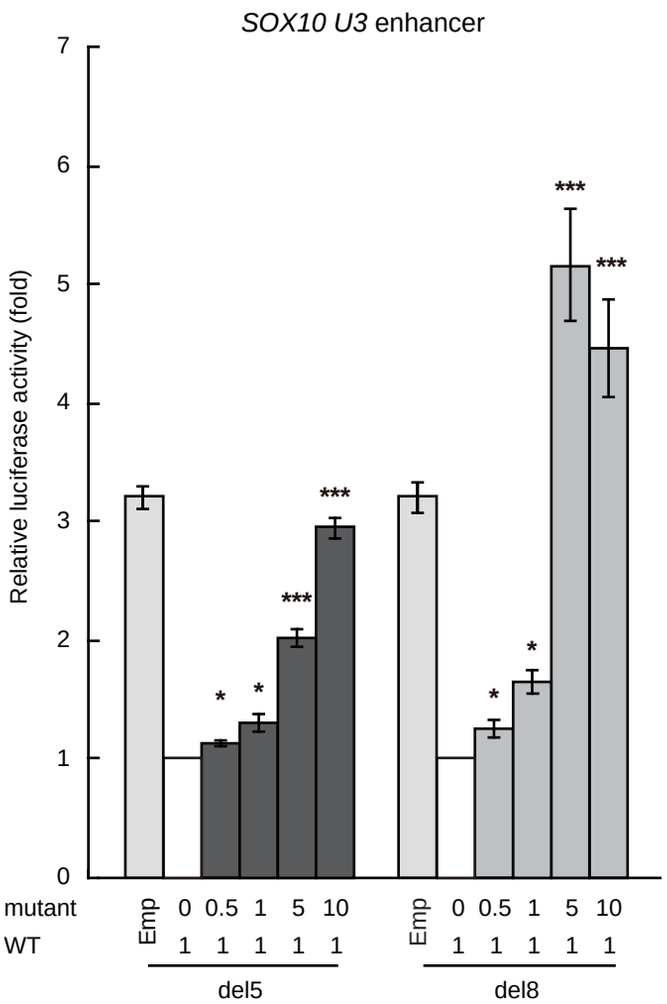

A
Gut
WT
del5/+
del8/+
B
Diamater (μm)
350
300
250
200
150
100
50
0
**
WT
del5
del8
C
Frequency (%)
3.5
3
2.5
2
1.5
1
0.5
0
WT
del5/+
del8/+
**
*
**
*
*
Primary
Passage 1
Passage 2
Passage 3
D
wt Phox2b
Sox10
Merge
WT
Phox2b del8/+
E
WT
Phox2b del5/+
Phox2b del8/+
Sox10 pH3
F
Tuj1
wt Phox2b
Sox10
Merge
WT
Phox2b del8/+

Supplemental Figure 4. Characterization of the enteric ganglion progenitors by a neuroshpere method
(A and B) Morphology and diameter of the primary neurospheres generated from embryonic gut (E13.5). (C) A graph showing neurosphere-forming ability. The numbers of neurospheres generated from 5,000 cells is shown as Frequency (%). Note that self-renewal ability was gradually declined and nearly completely lost after Passage 3 in NPARM *PHOX2B* mutant-derived neurosphere cells (D) Representative images for Phox2b/Sox10 expression in neurosphere cells cultured on monolayer. Unlike the sympathetic ganglion progenitors, the enteric ganglion progenitors of NPARM *PHOX2B* mutants were passage-able and displayed an apparently normal differentiation pattern at least in a few passages (Passage 1 shown). (E) Sox10 and phospho-histone H3 double labeling. Despite the apparently normal differentiation pattern shown in (D), there was a decrease in the ratio of double positive cells ($Sox10^{+}pH3^{+}/Sox10^{+}$) in mutant ENCC-derived neurosphere cells (Passage 1; wt vs. *del5* vs. *del8*; 18.9 ± 2.8% vs. 19.7 ± 3.8% vs. 14.2 ± 3.2%; $P = 0.7$ and 0.04 for wt vs. *del5* and wt vs. *del8*, respectively), revealing impaired proliferation of immature ENCCs of the mutant gut. (F) Immunocytochemical analysis of differentiating neurons. After longer passages, aberrant Sox10 expression was observed in neuronally differentiating cells ($Pho2b^{+}/TuJ1^{+}$) of mutant-derived neurosphere cells (Passage 5; compare $TuJ1^{+}$ cells depicted by arrows in top and bottom panels). Note also that neurites of $TuJ1^{+}$ cells in the mutant-derived neurosphere cells are shorter than those in wild type (white arrows in bottom panels). Scale bars: 20 μm in E and F; 40 μm in D; 200 μm in A. Error bars indicate SD ($n = 3$). Statistical significance: $*P < 0.05$, $**P < 0.01$



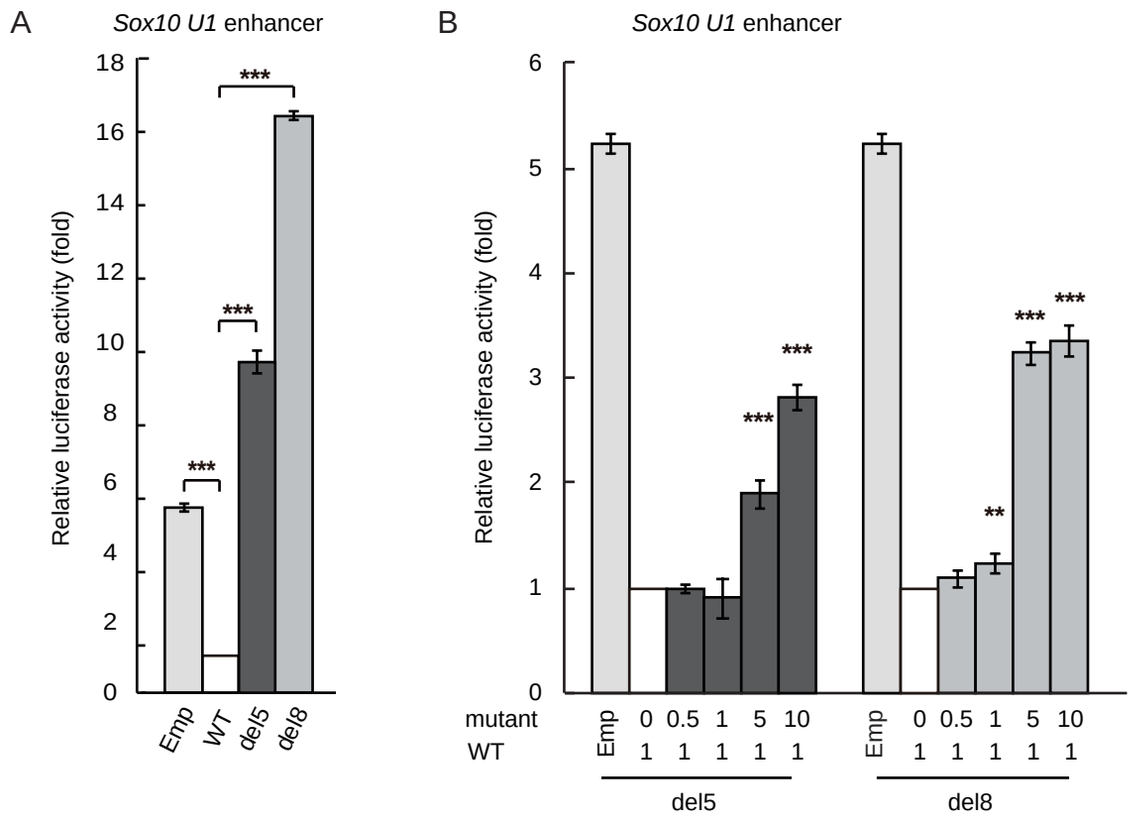
Supplemental Figure 5. Expression of wt and NPARM PHOX2B proteins in luciferase assays

Western blot analysis of FLAG-tagged wt and NPARM PHOX2B using anti-FLAG antibody is shown. Note that, in cells transfected with FLAG- wt PHOX2B or NPARM PHOX2B expression construct alone (lanes 1, 2 and 7, 8 for del5 and del8), expression levels of wt PHOX2B were higher than those of NPARM PHOX2B suggesting decreased stability of NPARM PHOX2B. An increase in NPARM PHOX2B protein levels was clearly observed corresponding to the increase in the amount of expression vectors transfected (lanes 3-6 and 9-12). Anti-α-tubulin antibody was used as a loading control.



Supplemental Figure 6. Interactions between wild type and NPARM PHOX2B on *Sox10 U3* enhancer

The graph shows the transactivation of the *U3* enhancer with a constant amount of wt PHOX2B expression construct and increasing amounts of NPARM PHOX2B expression constructs (from 1:0 to 1:10). Error bars indicate SD ($n = 3$). Statistical significance: *$P < 0.05$, ***$P < 0.001$



Supplemental Figure 7. Transcriptional effects of wt and NPARM PHOX2B on *Sox10 U1* enhancer

Transcriptional properties *Sox10 U1* reporter genes were examined in NIH3T3 cells using wt and NPARM PHOX2B expression constructs. (A) Similar to *U3*, wt PHOX2B showed repression, whereas NPARM PHOX2B exerted transactivation. (B) Interaction between wt and NPARM PHOX2B on *U1* enhancer. Although NPARM PHOX2B displayed inhibitory effects on repressive actions of wt PHOX2B, the net effect did not reach the levels of transactivation even at 1:10 (wt:NPARM PHOX2B) ratio. Error bars indicate SD (n = 3). Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$