eNeuro

Research Article: New Research | Development

Increased RET activity coupled with a reduction in the *RET* gene dosage causes intestinal aganglionosis in mice

https://doi.org/10.1523/ENEURO.0534-20.2021

Cite as: eNeuro 2021; 10.1523/ENEURO.0534-20.2021

Received: 8 December 2020
Revised: 24 March 2021
Accepted: 13 April 2021

Manuscript title:

Increased RET activity coupled with a reduction in the *RET* gene dosage causes intestinal aganglionosis in mice

Abbreviated Title: RET-activating mutation causing ENS deficits

Authors: Mitsumasa Okamoto, Toshihiro Uesaka, Keisuke Ito and Hideki Enomoto

Affiliation: Division for Neural Differentiation and Regeneration, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan

Author Contributions: TU and HE Designed Research; MO, TU and KI Performed Research: HE wrote the paper

Correspondence should be addressed to Hideki Enomoto at enomotoh@med.kobe-u.ac.jp

Number of Figures 5

Number of Tables 0

Number of Multimedia 0

Number of words of Abstract 178

Number of words for Significance of Statement: 115

Number of words for Introduction: 620

Number of words for Discussion: 810

Acknowledgements

The authors thank Hazuki Hiraga for editing this manuscript.

Conflict of Interest

The authors report no conflict of interest.

Funding sources

This work was supported by Japan Society for the Promotion of Science, Japan, Grant 17H03550, 17K19527, 16K08466, Grants-in-Aid for Scientific Research on Innovative

Areas “Stem Cell Aging and Disease” (#25115002) from MEXT, Takeda Science Foundation, and Yakult Bio-Science Foundation.

Abstract

Mutations of the gene encoding the *RET* tyrosine kinase causes Hirschsprung disease (HSCR) and medullary thyroid carcinoma (MTC). Current consensus holds that HSCR and MTC are induced by inactivating and activating RET mutations, respectively. However, it remains unknown whether activating mutations in the *RET* gene have adverse effects on ENS development in vivo. We addressed this issue by examining mice engineered to express RET51(C618F), an activating mutation identified in MTC patients. Although $Ret^{51(C618F)/51(C618F)}$ mice displayed hyperganglionosis of the ENS, $Ret^{51(C618F)/-}$ mice exhibited severe intestinal aganglionosis due to premature neuronal differentiation. Reduced levels of GDNF, a RET-activating neurotrophic factor, ameliorated the ENS phenotype of $Ret^{51(C618F)/-}$ mice, demonstrating that GDNF-mediated activation of RET51(C618F) is responsible for severe aganglionic phenotype. The *RET51(C618F)* allele showed genetic interaction with *Ednrb* gene, one of modifier genes for HSCR. These data reveal that proliferation and differentiation of ENS precursors are exquisitely controlled by both the activation levels and total dose of RET. Increased RET activity coupled with a decreased gene dosage can cause intestinal

aganglionosis, a finding that provides novel insight into HSCR pathogenesis.

SIGNIFICANCE STATEMENT

Mutations of the *RET* gene have been identified in Hirschsprung disease (HSCR) and neuroendocrine tumors (NET). It has been thought that HSCR and NET are caused by inactivating and activating mutations of the RET gene, respectively. However, little is known about whether enhanced RET activity exerts any roles in the pathogenesis of HSCR. We show that mice carrying an activating mutation in the *Ret* gene display intestinal aganglionosis when the *Ret* gene dosage is halved. The aganglionosis phenotype is caused by premature neuronal differentiation and impaired migration of ENS precursors These findings raise the possibility that RET-activating mutations can cause HSCR when associated with a reduction in the dosage or expression of the *RET* gene.

Introduction

RET is a receptor tyrosine kinase that serves as a signaling receptor for the glial cell-derived neurotrophic factor (GDNF) family ligands (GFLs) (Baloh et al., 2000; Airaksinen and Saarma, 2002). Binding of GFLs to their cognate GFRα receptors induces dimerization and subsequent autophosphorylation of RET, culminating in the activation of downstream intracellular signaling. RET is expressed in a wide variety of neural crest (NC)-derived cell types and endoderm-derived thyroid C cells. In humans, mutations in the *RET* gene are associated with the pathogenesis of various forms of diseases that include Hirschsprung's disease (HSCR) and medullary thyroid carcinoma (MTC). Hereditary MTCs occurs in multiple endocrine neoplasia (MEN) 2, which is further subcategorized into MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC) based on the phenotype such as pheochromocytoma, hyperparathyroidism and/or other developmental anomalies (infertility, marfanoid habitus etc.) (Wells et al., 2013; Tomuschat and Puri, 2015).

HSCR is characterized by the congenital loss of enteric ganglia in the distal portion of the gut (intestinal aganglionosis). Genetic studies have revealed that HSCR is a multifactorial disease that involves mutations in multiple genes for its pathogenesis and exhibits complex patterns of inheritance. To date, mutations have been identified in as

88 many as 17 genes (Tilghman et al., 2019), and, among which the RET gene is most
89 frequently mutated (Amiel et al., 2008). HSCR-associated RET mutations have been
90 identified throughout the *RET* genome, affecting both coding and non-coding regions.
91 Coding mutations account for only 50% of familial and 15% of sporadic cases of HSCR
92 (Edery et al., 1994; Romeo et al., 1994). Meanwhile, some non-coding variants that
93 potentially affect the enhancer activity of the RET gene are considered necessary, albeit
94 not sufficient, mutations in isolated HSCR cases (Kapoor et al., 2015). Thus, HSCR is a
95 complex genetic trait in which reduced RET expression confers susceptibility to the
96 disease.

97 In contrast to HSCR, MEN2 displays rather simple genetic features. Most of those
98 mutations affects a restricted cysteine residue in the extracellular domain of RET,
99 converting it to arginine, tyrosine or phenylalanine. These amino acid conversions
100 disrupt intra-molecular cysteine bonding and causes aberrant inter-molecular bonding
101 and successive auto-phosphorylation of RET, leading to its aberrant and
102 ligand-independent activation. Those RET mutations are likely sufficient for the
103 development of tumors because familial MEN2 cases demonstrate autosomal dominant
104 inheritance (Margraf et al., 2009).

105 Together, HSCR- and MTC-associated RET mutations display distinct features, and,

106 although the whole spectrum of biological effects by those RET mutations have not
107 been fully elucidated, current consensus holds that HSCR is caused by inactivating
108 mutations of the *RET* gene whereas MTC is induced by activating mutations of RET
109 (Hansford and Mulligan, 2000).

110 Although previous studies support that inactivation or downregulation of RET signaling
111 leads to HSCR-like intestinal aganglionosis in mice, it has not been clear whether
112 activating mutations of RET have adverse effects on ENS development (Amiel et al.,
113 2008). To address this issue, we examined the development of the ENS in mice
114 engineered to express RET C618F, one of the MTC-associated RET-activating mutants,
115 under the endogenous *Ret* promoter (Okamoto et al., 2019). Biochemical studies
116 revealed that RET C618F displays slightly higher RET basal phosphorylation than
117 normal, but still requires GDNF for its full activation (Okamoto et al., 2019). Thus, RET
118 C618F mutant mice are an ideal platform to understand how the ENS develops when
119 the activity of RET is slightly elevated. We found that, in mice carrying the RET C618F
120 mutation, the ENS phenotype changed dramatically from hyperganglionosis to
121 aganglionosis when the Ret gene dosage was changed from two copies to one copy.
122 Premature neuronal differentiation of ENS precursors contributed to the aganglionosis
123 phenotype. Our findings reveal a novel mechanism of HSCR pathogenesis that is

124 Ret-activating mutations can cause HSCR when the Ret gene dosage is reduced.

125

Methods

Mice

The generation and characterization of *Ret51* and *Ret51*(C618F) mice have been described previously (Okamoto et al., 2019). We obtained Ret^{GFP} (a kind gift from J. Milbrandt, Washington University School of Medicine (Jain et al., 2006), $Gdnf^{+/-}$ (a kind gift from V. Pachnis The Francis Crick Institute, London, UK (Moore et al., 1996), and $Ednrb^{flex3}$ mice (a kind gift from M.L. Epstein, University of Wisconsin-Madison (Druckenbrod et al., 2008)). $Ednrb^{+/-}$ mice were obtained by crossing $Ednrb^{flex3}$mice to *Actb::Cre* mice (Stock No: 019099; Jackson Laboratories (Lewandoski et al., 1997). Mice were bred and maintained at the Institute of Experimental Animal Research of Kobe University Graduate School of Medicine under specific pathogen_free conditions and all animal experiments were performed according to the Kobe University Animal Experimentation Regulations.

Whole-mount immunostaining

Dissected gut from embryos or P0 pups were fixed with 4% paraformaldehyde (PFA) in PBS containing 10mM phosphate buffer, pH7.4, 137mM sodium chloride, and 2.7 mM potassium chloride overnight at 4°C and incubated in 1% Triton X-100 in PBS for 30

144 min at room temperature. After fixation and permeabilization, the preparations were
145 incubated in 0.1 M glycine in PBS for 2-6 hours and processed for
146 immunohistochemistry. For the preparations from P0 pups, blocking solution contains
147 5% skim milk, 5% DMSO, 1% Tween20 in PBS. The following antibodies were used:
148 guinea pig anti-Phox2b (1:1000, home-made, raised against the C-terminal region of
149 Phox2b (RRID:AB_2313690) (Pattyn et al., 1997); goat anti-Sox10 (1:300,
150 Cat.#sc-17342, Santa Cruz Biotechnology Inc., RRID: AB_2195374); rabbit
151 anti-PGP9.5 (1:1000, Cat.#RA-95101, Ultra Clone, RRID: AB_2313685); rabbit
152 anti-phospho-ERK1/2 (1:500, Cat.#9101, Cell signaling Technology, RRID:
153 AB_331646) and chicken anti-GFP (1:1000, Cat.#GFP-1020, Aves Laboratories, RRID:
154 AB_10000240). We used the following secondary antibodies (Biotium): CF488A
155 donkey anti-rabbit IgG (Cat.#20015, RRID: AB_10559669), CF488A donkey
156 anti-chicken IgY (Cat.#20166, RRID: AB_10854387), CF568 donkey anti-guinea-pig
157 IgG (Cat.#20377), CF568 donkey anti-goat IgG (Cat.#20106, RRID: AB_10559672),
158 CF568 donkey anti-rabbit IgG (Cat.#20098, RRID: AB_10557118), and CF640T
159 donkey anti-goat IgG (Cat.#20179, RRID: AB_10853145).
160
161 *Whole_mount gut EdU assays.*

E12.5 or 14.5 pregnant females were injected intraperitoneally with 5-ethynyl-2'-deoxyuridine (EdU) (50 µg/g body weight). Two hours after injection, dissection of gut was followed by fixation and permeabilization in the same fashion as whole_mount immunostaining. The preparations were washed twice for 3 min with 3% BSA in PBS at room temperature. For EdU assays (Click_iT Plus EdU Imaging Kit, Invitrogen), the reaction cocktail (reaction buffer, $CuSO_4$, Alexa Flour 594 azide and buffer additive as per manufacture's protocol) was added for 30 min followed by rinsing twice for 3 min with 3% BSA in PBS in the dark at the room temperature. After EdU labeling, whole_mount immunostaining was performed as described above.

Cell counts

$Phox2b^+$ neurons were counted on ten sections per investigated region of the gut at P0. $Phox2b^+$ and EdU^+ $Phox2b^+$ ENS precursors were counted in a minimum of five areas at even intervals of the midgut longitudinally (0.025 mm^2 each) and the rate of ENS precursor proliferation was determined in animals for each genotype.

Experimental design and statistical analysis

Images were carefully selected to show the average effect obtained for each

experimental condition. All descriptive statistics are presented as means ± SEM. Normality of the data was tested with Levene's test (Dr.SPSS II Statistics software (SPSS Inc, IL, USA)), and differences were subsequently assessed using unpaired t-test. If assumptions for a parametric test were not met (Levene's test, $p < 0.05$), unpaired t test with Welch correction was used. Statistical analyses for enteric neuron numbers were performed using one-way ANOVA, followed by pairwise comparisons (Tukey's *post hoc* test) where appropriate. GraphPad Prism 5 software (GraphPad Software Inc. CA, USA) was used to conduct unpaired t-test with Welch correction, one-way ANOVA with Tukey test and Chi-square test. Animals of both sex were used. No methods were used for sample size determination.

Results

RET (C618F) enhances proliferation of ENS precursors and causes intestinal hyperganglionosis

To understand the biological impact of enhanced RET signaling on ENS development, we examined mice expressing RET(C618F), a MEN2-associated RET-activating mutant. Previous studies revealed that, among MEN2-associated RET mutants, those affecting RET(C618) residues display moderate to low transforming activity in vitro (Carlomagno et al., 1997; Ito et al., 1997). Our biochemical characterization indicated that RET(C618F) displays slightly higher basal phosphorylation than normal and requires GDNF for its full phosphorylation (Okamoto et al., 2019). Because RET(C618F) retains GDNF-responsiveness and exhibits moderate activation of RET-signaling, RET(C618F) is an ideal RET mutant to examine the effect of slight RET-signaling enhancement on ENS development. Since mice expressing RET(C618F) were engineered to express RET51(long isoform) cDNA carrying a C618F mutation by the endogenous *Ret* promoter, the mutant allele is hereafter referred to as 51(C618F). As a control, mice expressing wild type *RET51* cDNA were used (the allele referred to as 51). $Ret^{51(C618F)/51(C618F)}$ mice were born apparently normally at an expected Mendelian ratio but all died of unknown causes within 24 hours after birth (Okamoto et al., 2019).

In newborn (P0) $Ret^{51(C618F)/51(C618F)}$ mice, histological analysis of the gut revealed that the density of enteric neurons in the myenteric layer of the small intestine appeared higher in $Ret^{51(C618F)/51(C618F)}$ mice than $Ret^{51/51}$ mice (control, Fig. 1A). Neuronal count confirmed a significant increase in the numbers of myenteric neurons in both small intestine and colon of $Ret^{51(C618F)/51(C618F)}$ mice ($p < 0.0001$, Fig.1B).

We investigated proliferation of ENS precursors by anti-Phox2b staining (which detects almost all ENS precursors during mid-gestation) combined with EdU labeling at embryonic day 12.5 (E12.5: a period of ENS precursor migration) and E14.5 (a period when ENS precursor migration is completed). This analysis revealed an increase in double-positive cell populations in $Ret^{51(C618F)/51(C618F)}$ embryos as compared to $Ret^{51/51}$ embryos in both of these developmental periods (Fig. 1C and D, $Ret^{51(C618F)/51(C618F)}$ vs. $Ret^{51/51}$ in the midgut; 43.3±2.9% vs. 31.7±0.6% ($p = 0.019$) at E12.5 and 13.9±2.1% vs. 10.2±0.9% ($p = 0.046$) at E14.5, respectively). Thus, the increase in enteric neuron numbers in newborn $Ret^{51(C618F)/51(C618F)}$ mice is attributed at least in part to enhanced proliferation of ENS progenitors

Previous studies suggested that reduced RET signaling impairs ENS migration (Young et al., 2001; Natarajan et al., 2002; Uesaka et al., 2008) and that proliferation of ENS precursors is a major driving force for ENS migration (Landman et al., 2007). We

228 therefore examined the migration of ENS precursors in *Ret*$^{51(C618F)/51(C618F)}$ embryos.
229 Unexpectedly, the migratory wavefront of ENS precursors was always slightly delayed
230 in *Ret*$^{51(C618F)/51(C618F)}$ embryos as compared to control embryos at E12.5 (Fig. 2, upper
231 panel). However, this delay was only transient and compensated for before birth. The
232 ENS was fully developed in all of *Ret*$^{51(C618F)/51(C618F)}$ neonates (Fig. 2, lower panel).
233 In adult *Ret*$^{51(C618F)/+}$ mice, we detected focal hyperplasia of thyroid C cells (Okamoto et
234 al., 2019), a pre-cancerous condition that leads to medullary thyroid carcinoma (Wolfe
235 et al., 1973). Together, these results indicate that, consistent with its enhanced activity in
236 vitro, RET51 (C618F) confers gain-of-function effects on development of the ENS and
237 thyroid C cells.
238
239 **_Ret_$^{51\ (C618F)/-}$ mice display intestinal aganglionosis**
240 We moved on to examine the effects of a reduction in the dosage of the *RET* gene
241 because reduced RET expression is known to confer susceptibility to intestinal
242 aganglionosis in both human and mice (Emison et al., 2005; Uesaka et al., 2008). We
243 crossed *Ret*$^{51/51}$ or *Ret*$^{51(C618F)/51(C618F)}$ mice to *Ret*$^{EGFP/+}$ mice in which one of the *Ret*
244 alleles was replaced by the *Ret-EGFP* allele (*Ret* null). Consistent with previous
245 observations that one allele of wild-type RET-expressing allele is sufficient for normal

development of the ENS in mice, the gut was fully furnished with ENS meshwork in *Ret*$^{51/EGFP}$ mice (Fig.3A and B left). In a stark contrast, all of *Ret*$^{51(C618F)/EGFP}$ mice displayed intestinal aganglionosis (Fig 3B). This result was surprising, as *Ret*$^{51(C618F)/51(C618F)}$ mice display hyperganglionosis (Fig.1A). Although the length of aganglionic gut was varied, in about 62% of *Ret*$^{51(C618F)/EGFP}$ mice (29 out of 34 mice examined), the ENS was present only in the small intestine (Fig. 3C). Among these mice, 8 mice (20% of all examined) displayed skip segment-type aganglionosis (Fig. 3B, third picture). This skip segment appears to be developed at least partially due to impaired migration of trans-mesenteric ENS progenitors, a cell population primarily contributing to colonic ENS (Nishiyama et al., 2012), because we occasionally found a limited number of enteric neurons scattered in the colon in some of *Ret*$^{51(C618F)/EGFP}$ embryos at E13.5, a period 2 days after trans-mesenteric migration is completed (Fig. 4). These data demonstrate that *RET51*(C618F) allele causes severe intestinal aganglionosis when the *RET* gene dosage is reduced to half.

Premature neuronal differentiation impairs migration of ENS progenitors in *Ret*$^{51(C618F)/EGFP}$ embryos

To investigate the mechanism underlying the impaired ENS development in

264 *Ret*$^{51(C618F)/EGFP}$ mice, we conducted whole-mount immunohistochemical analyses of
265 embryonic gut (E12.5). In control (*Ret*$^{51/EGFP}$) embryos, ENS progenitors at the
266 migrating wavefront invaded the proximal colon and expressed both RET (revealed by
267 GFP fluorescence) and Sox10 (Fig. 5A, left), indicating those cells are immature
268 progenitors. Consistent with this expression pattern, none of the cells at the wavefront
269 expressed PGP9.5 (Fig. 5B, left), a marker for neuronal differentiation. In contrast, in
270 *Ret*$^{51(C618F)/EGFP}$ embryos, Sox10 expression was lost in many cells at the wavefront (Fig.
271 5A, right, arrowheads). Associated with this change, we found aberrant expression of
272 PGP9.5 in ENS progenitors at the migratory wavefront, which was located primarily in
273 the midgut (Fig. 5B, right). These results indicate that premature neuronal
274 differentiation is induced in ENS progenitors at the migratory wavefront in
275 *Ret*$^{51(C618F)/EGFP}$ embryos.
276 A previous study revealed that elevation of ERK activity is associated with induction of
277 neuronal differentiation in ENS progenitors (Uesaka et al., 2013). We examined ERK
278 activation by whole-mount staining of embryonic gut (E12.5) using anti-phospho Erk
279 (pErk) antibodies. In *Ret*$^{51/EGFP}$ embryos, pErk-positive ENS progenitors were abundant
280 in proximal regions of the midgut, whereas such cells were almost undetectable at the
281 wavefront region (Fig. 4C, right upper panels). In contrast, pErk-positive cells were

frequently observed not only in the proximal midgut but also in the wavefront regions in *Ret*$^{51(C618F)/EGFP}$ embryos (Fig. 5C, left and right bottom panels). These data collectively indicate that single allele-only expression of RET51 (C618F) causes premature enteric neuronal differentiation in vivo.

GDNF-mediated activation of RET51(C618F) is responsible for premature neuronal differentiation of ENS progenitors

Previous biochemical analyses revealed that RET51(C618F) responds to GDNF and displays enhanced phosphorylation in vitro. To investigate whether GDNF-induced stimulation of RET51(C618F) contributes to severe aganglionosis phenotype in *Ret*$^{51(C618F)/+}$ mice, we examined whether severity of the phenotype changes in *Ret*$^{51(C618F)/EGFP}$ mice on *Gdnf*$^{+/-}$ background. By whole-mount GFP staining of the neonatal gut (n= 20), we found that most of *Ret*$^{51(C618F)/EGFP}$ /*Gdnf*$^{+/-}$ mice (80%) displayed colonic aganglionosis (Fig. 6A), which stood in a sharp contrast to the ENS phenotype of *Ret*$^{51(C618F)/EGFP}$ that showed mostly extensive aganglionosis (aganglionic segment exceeding to the small intestine). Chi- square test of independence confirmed the significant differences between *Ret*$^{51(C618F)/EGFP}$ /*Gdnf*$^{+/-}$ and *Ret*$^{51(C618F)/EGFP}$ /*Gdnf*$^{+/+}$ mice ($p = 0.005 < 0.01$). Interestingly, in one case, the ENS was found fully

300 developed up to the anal end (Fig. 6B). Moreover, skip segment-type aganglionosis,
301 which was identified in 24% of $Ret^{51\ (C618F)/\ -}$ mice, was not detected in any of
302 $Ret^{51(C618F)/EGFP}$ /$Gdnf^{+/-}$ mice. These results collectively indicate that reduction in
303 GDNF levels exerts significant rescue effects on severe aganglionosis phenotype (Fig.
304 6A-B).
305 At E12.5, migration of ENS precursors was delayed in $Ret^{51(C618F)/EGFP}$/$Gdnf^{+/-}$ embryos
306 as compared to $Ret^{51/EGFP}$ /$Gdnf^{+/-}$embryos (Fig. 6C). To examine the effect of the
307 reduction of *Gdnf* gene dosage on intracellular signaling, whole-mount pERK staining
308 was performed on the gut of $Ret^{51(C618F)/EGFP}$/$Gdnf^{+/-}$ embryos (E12.5). Similar to wild
309 type or $Ret^{51/EGFP}$ embryos (Fig. 4C top), ERK phosphorylation was undetectable at the
310 wavefront regions of $Ret^{51(C618F)/EGFP}$/$Gdnf^{+/-}$ embryos (Fig. 6D, right). These data reveal
311 that GDNF-mediated activation of RET51(C618F) is responsible for aberrant
312 phosphorylation of ERK in ENS precursors at the wavefront and causes intestinal
313 aganglionosis in $Ret^{51\ (C618F)/\ -}$ embryos.
314
315 **An allelic loss of the *Ednrb* gene exacerbates the ENS phenotype of $Ret^{51(C618F)/-}$**
316 **mice**
317 Previous studies revealed a genetic interaction between the *Ret* and *Ednrb* genes in

318 HSCR pathogenesis. Either *Ret* heterozygosity or the *Ednrb ls/ls* allele alone exerts no
319 adverse effect on ENS development, but induces severe intestinal aganglionosis when
320 combined (Carrasquillo et al., 2002). Ednrb signaling regulates multiple processes of
321 ENS development including migration, proliferation and differentiation of ENS
322 precursors (Barlow et al., 2003; Kruger et al., 2003). We examined a potential genetic
323 interaction between the *Ret51(C618F)* allele and the *Ednrb* gene by crossing
324 $Ret^{51/51(C618F)}$ mice to $Ednrb^{+/-}/Ret^{EGFP/+}$ mice. We found that, in contrast to
325 $Ret^{51/EGFP}/Ednrb^{+/-}$ embryos, which displayed normal ENS development,
326 $Ret^{51(C618F)/EGFP}$ / $Ednrb^{+/-}$ embryos exhibited severe intestinal aganglionosis in which
327 the ENS is observed only in the proximal part of the small intestine (Fig. 7A-C).
328 Immunohistochemical examination of ENS precursors revealed robust phosphorylation
329 of ERK and loss of Sox10 expression at the wavefront regions (Fig. 7D). Thus reduction
330 of one copy of the *Ednrb* gene leads to exacerbation of the aganglionosis phenotype,
331 which contrasted the ameliorating effect by the reduction of the *Gdnf* gene dosage. This
332 difference is not caused by the differential expression levels of GFRα1, the cognate
333 receptor for GDNF involved in HSCR pathogenesis (Lui et al., 2002), as it was
334 expressed at comparable levels in $Gdnf^{+/-}$, $Ednrb^{+/-}$ and wild type embryos (Fig. 7E). At
335 any rate, these results reveal a clear genetic interaction between the *Ret51(C618F)* allele

and the *Ednrb* gene and suggest that the Ednrb signaling functions to inhibit premature differentiation of ENS precursors in this context.

Discussion

In this study, we have provided evidence that RET(C618F), a RET-activating mutant, causes intestinal aganglionosis when the *Ret* gene copy number is reduced to half, which is contrary to the current consensus that enteric aganglionosis is caused by inactivating RET mutations. This unexpected finding provides novel insights into mechanisms underlying the development of the ENS by RET/GDNF signaling and the pathogenesis of HSCR.

The involvement of RET activating mutations in HSCR was first described in co-segregation of MEN2A/FMTC and HSCR in a fraction of families. These patients carry missense mutations in the *RET* gene, which substitutes arginine or serine for a cysteine residue at position 618 or 620. These RET mutants display ligand-independent constitutive activation due to inter-molecular di-sulfide-linked dimerization (Santoro et al., 1995) and simultaneously loses cell surface expression (Asai et al., 1995). The former property contributes to neoplastic pathology including MTC and pheochromocytoma, whereas the latter contributes to impaired development of the ENS (HSCR). Therefore, in the context of ENS development, these mutants (C618R, C618S and C620R) behave as RET-inactivating mutants (Mulligan et al., 1994; Borst et al., 1995). Animal studies support this notion, as mice harboring RET(C620R) mutation in a

358 homozygous fashion display kidney agenesis and intestinal aganglionosis, a phenotype
359 identical to that of *Ret*-deficient mice (Carniti et al., 2006; Yin et al., 2007).
360 RET(C618F) examined in this study exhibited distinct properties. Unlike other C618 or
361 C620 mutants, RET(C618F) is expressed on the cell surface (Okamoto et al., 2019).
362 Although phosphorylation levels of RET(C618F) are slightly higher than those of wild
363 type RET, GDNF stimulation further enhances phosphorylation of RET(C618F)
364 (Okamoto et al., 2019). Thus, RET(C618F) is a GDNF-responsive RET-activating
365 mutant. Consistent with these biochemical properties, $Ret^{51(C618F)/51(C618F)}$ mice had
366 increased numbers of enteric neurons due to enhanced proliferation of ENS precursors.
367 Surprisingly, despite the activating nature of RET51(C618F), $Ret^{51(C618F)/-}$ mice
368 displayed severe intestinal aganglionosis, in sharp contrast to $Ret^{RET51/-}$ mice (control),
369 which exhibited no ENS deficit. This study provides evidence, for the first time to our
370 knowledge, that RET-activating mutations can cause intestinal aganglionosis when
371 coupled with a reduction in the *Ret* gene dosage.

372 It is important to note that, the *RET C618F* allele displays genetic interaction
373 with the *Ednrb* gene, which is known as a modifier gene for HSCR carrying mutations
374 in the *RET* gene. Our findings suggest a novel pathogenetic mechanisms of HSCR by
375 revealing how reduced RET expression affects ENS development and confers

susceptibility to HSCR (Emison et al., 2005). It is also important to note that many of *Ret*$^{51(C618F)/-}$ mice displayed skip-segment aganglionosis. *Ret*$^{51(C618F)/-}$ mice thus serve as the first valuable platform to investigate the molecular and cellular mechanisms underlying this mysterious condition.

Histological examination of *Ret*$^{51(C618F)/-}$ embryos revealed that premature neuronal differentiation of ENS precursors is likely to be the cause of the intestinal aganglionosis. Exacerbation of the aganglionic phenotype by the reduction of the *Ednrb* gene supports this possibility because endothelin-3/Ednrb signaling prevents premature neuronal differentiation (Wu et al., 1999). The aganglionic phenotype of *Ret*$^{51(C618F)/-}$ embryos stands in sharp contrast to that of *Ret*$^{51(C618F)/51(C618F)}$ embryos, in which ENS precursors underwent proliferation rather than differentiation. Although the exact mechanism by which ENS precursors adopt to a different cell fate (proliferation or differentiation) is unknown, it may involve regulation of Erk phosphorylation. In PC12 cells, EGF treatment enhances cell proliferation, while FGF treatment induces neuronal differentiation. This difference in cell fate is tightly associated with the levels and kinetics of Erk phosphorylation. EGF evokes a rapid surge and subsequent abrupt quenching of Erk phosphorylation, whereas FGF induces long-lasting and moderate levels of Erk phosphorylation (Qiu and Green, 1991; Traverse et al., 1992; Nguyen et al.,

1993). Interestingly, Erk phosphorylation-associated cell fate determination in ENS precursors was reported previously (Natarajan et al., 2002; Asai et al., 2006; Goto et al., 2013) . We can therefore assume that Erk activity is differentially regulated in ENS precursors between *Ret*$^{51(C618F)/51(C618F)}$ and *Ret*$^{51(C618F)/-}$ embryos. We tried to examine this possibility by culturing ENS precursors and conducting biochemical analyses. Unfortunately, however, *Ret*$^{51(C618F)/-}$ ENS precursors displayed a tendency to differentiate in vitro, and we were unable to obtain reliable data. To understand the mechanisms underlying the intestinal aganglionosis in *Ret*$^{51(C618F)/-}$ mice, we also have to understand the biochemical properties of RET51(C618F) in more detail. Although RET51(C618F) is expressed on cell surface, it is also detected in the cytoplasm (Okamoto et al., 2019). The latter likely reflects localization in ER, which is commonly observed in all MEN-associated RET mutant proteins (Wagner et al., 2012). Thus, RET51(C618F) has combined properties of wild type RET and MEN-associated RET mutants. Even on the cell surface, it is unknown whether RET51(C618F) behaves as wild type RET. For instance, upon GDNF binding to GFRα receptors, wild type RET protein gets recruited to the raft and phosphorylated, which provides a platform to activate Src and Akt-PI3 kinase efficiently. It is currently unknown how RET51(C618F) is localized and activates intracellular signaling molecules on the cell surface. In these

412 respects, RET51(C618F) may not reflect enhanced activity of purely wild type RET.
413 RET51(C618F) has unique biochemical properties among MEN-associated RET
414 mutants, which suggest that all of RET-activating mutations do not necessarily cause the
415 intestinal aganglionosis by *RET* gene dosage reduction.

416 Amelioration of the phenotype in $Ret^{51(C618F)/-}$ embryos by the *Gdnf* gene
417 reduction indicates that GDNF-mediated activation of RET51(C618F) is responsible for
418 the severe aganglionic phenotype. Although NRTN also activates RET in developing
419 ENS (Heuckeroth et al., 1998), contribution of NRTN-mediated RET(C618F) activation
420 to the aganglionic phenotype is unlikely because expression of GFRα2, the cognate
421 receptor for NRTN, occurs later than a period when the aganlionic phenotype in
422 $Ret^{51(C618F)/-}$ embryos becomes obvious. It is important to note that, in normal ENS
423 development, enteric neuron numbers are determined primarily by the levels of GDNF
424 signaling. Mice heterozygous for the GDNF-deficient allele ($Gdnf^{+/-}$ mice) display
425 reduced numbers of enteric neurons (Gianino et al., 2003). In contrast, reduction of
426 Sprouty2, an inhibitor of Erk phosphorylation downstream of RET/GDNF signaling,
427 leads to hyperganglionosis of the gut (Taketomi et al., 2005). This hyperganglionosis
428 phenotype is suppressed on $Gdnf^{+/-}$ background. Evidence also suggests that RET
429 expression is regulated by RET activity induced by GDNF (Oppenheim et al., 2000).

Taken together, both signaling and expression of RET are exquisitely controlled by the availability of GDNF, Sprouty2 and phosphor-Erk. Even a slight disturbance (both upregulation and downregulation) of RET signaling can abrogate ENS development (Nagy et al., 2020). Understanding the development and developmental disorders of the ENS requires the elucidation of interactions among these molecules.

436

437 **References**

438 Airaksinen MS, Saarma M (2002) The GDNF family: signalling, biological functions
439 and therapeutic value. Nat Rev Neurosci 3:383-394.

440 Amiel J et al. (2008) Hirschsprung disease, associated syndromes and genetics: a review.
441 Journal of medical genetics 45:1-14.

442 Asai N, Iwashita T, Matsuyama M, Takahashi M (1995) Mechanism of activation of the
443 ret proto-oncogene by multiple endocrine neoplasia 2A mutations. Molecular
444 and cellular biology 15:1613-1619.

445 Asai N, Fukuda T, Wu Z, Enomoto A, Pachnis V, Takahashi M, Costantini F (2006)
446 Targeted mutation of serine 697 in the Ret tyrosine kinase causes migration
447 defect of enteric neural crest cells. Development (Cambridge, England)
448 133:4507-4516.

449 Baloh RH, Enomoto H, Johnson EM, Jr., Milbrandt J (2000) The GDNF family ligands
450 and receptors - implications for neural development. Curr Opin Neurobiol
451 10:103-110.

452 Barlow A, de Graaff E, Pachnis V (2003) Enteric nervous system progenitors are
453 coordinately controlled by the G protein-coupled receptor EDNRB and the
454 receptor tyrosine kinase RET. Neuron 40:905-916.

455 Borst MJ, VanCamp JM, Peacock ML, Decker RA (1995) Mutational analysis of
456 multiple endocrine neoplasia type 2A associated with Hirschsprung's disease.
457 Surgery 117:386-391.
458 Carlomagno F, Salvatore G, Cirafici AM, De Vita G, Melillo RM, de Franciscis V,
459 Billaud M, Fusco A, Santoro M (1997) The different RET-activating capability
460 of mutations of cysteine 620 or cysteine 634 correlates with the multiple
461 endocrine neoplasia type 2 disease phenotype. Cancer research 57:391-395.
462 Carniti C, Belluco S, Riccardi E, Cranston AN, Mondellini P, Ponder BA, Scanziani E,
463 Pierotti MA, Bongarzone I (2006) The Ret(C620R) mutation affects renal and
464 enteric development in a mouse model of Hirschsprung's disease. The American
465 journal of pathology 168:1262-1275.
466 Carrasquillo MM, McCallion AS, Puffenberger EG, Kashuk CS, Nouri N, Chakravarti A
467 (2002) Genome-wide association study and mouse model identify interaction
468 between RET and EDNRB pathways in Hirschsprung disease. Nature genetics
469 32:237-244.
470 Druckenbrod NR, Powers PA, Bartley CR, Walker JW, Epstein ML (2008) Targeting of
471 endothelin receptor-B to the neural crest. Genesis 46:396-400.
472 Edery P, Lyonnet S, Mulligan LM, Pelet A, Dow E, Abel L, Holder S, Nihoul-Fekete C,

473 Ponder BA, Munnich A (1994) Mutations of the RET proto-oncogene in
474 Hirschsprung's disease. Nature 367:378-380.
475 Emison ES, McCallion AS, Kashuk CS, Bush RT, Grice E, Lin S, Portnoy ME, Cutler
476 DJ, Green ED, Chakravarti A (2005) A common sex-dependent mutation in a
477 RET enhancer underlies Hirschsprung disease risk. Nature 434:857-863.
478 Gianino S, Grider JR, Cresswell J, Enomoto H, Heuckeroth RO (2003) GDNF
479 availability determines enteric neuron number by controlling precursor
480 proliferation. Development (Cambridge, England) 130:2187-2198.
481 Goto A, Sumiyama K, Kamioka Y, Nakasyo E, Ito K, Iwasaki M, Enomoto H, Matsuda
482 M (2013) GDNF and endothelin 3 regulate migration of enteric neural
483 crest-derived cells via protein kinase A and Rac1. J Neurosci 33:4901-4912.
484 Hansford JR, Mulligan LM (2000) Multiple endocrine neoplasia type 2 and RET: from
485 neoplasia to neurogenesis. Journal of medical genetics 37:817-827.
486 Heuckeroth RO, Lampe PA, Johnson EM, Milbrandt J (1998) Neurturin and GDNF
487 promote proliferation and survival of enteric neuron and glial progenitors in
488 vitro. Developmental biology 200:116-129.
489 Ito S, Iwashita T, Asai N, Murakami H, Iwata Y, Sobue G, Takahashi M (1997)
490 Biological properties of Ret with cysteine mutations correlate with multiple

endocrine neoplasia type 2A, familial medullary thyroid carcinoma, and Hirschsprung's disease phenotype. Cancer research 57:2870-2872.

Jain S, Encinas M, Johnson EM, Jr., Milbrandt J (2006) Critical and distinct roles for key RET tyrosine docking sites in renal development. Genes & development 20:321-333.

Kapoor A, Jiang Q, Chatterjee S, Chakraborty P, Sosa MX, Berrios C, Chakravarti A (2015) Population variation in total genetic risk of Hirschsprung disease from common RET, SEMA3 and NRG1 susceptibility polymorphisms. Human molecular genetics 24:2997-3003.

Kruger GM, Mosher JT, Tsai YH, Yeager KJ, Iwashita T, Gariepy CE, Morrison SJ (2003) Temporally distinct requirements for endothelin receptor B in the generation and migration of gut neural crest stem cells. Neuron 40:917-929.

Landman KA, Simpson MJ, Newgreen DF (2007) Mathematical and experimental insights into the development of the enteric nervous system and Hirschsprung's disease. Development, growth & differentiation 49:277-286.

Lewandoski M, Meyers EN, Martin GR (1997) Analysis of Fgf8 gene function in vertebrate development. Cold Spring Harb Symp Quant Biol 62:159-168.

Lui VC, Samy ET, Sham MH, Mulligan LM, Tam PK (2002) Glial cell line-derived

neurotrophic factor family receptors are abnormally expressed in aganglionic bowel of a subpopulation of patients with Hirschsprung's disease. Lab Invest 82:703-712.

Margraf RL, Crockett DK, Krautscheid PM, Seamons R, Calderon FR, Wittwer CT, Mao R (2009) Multiple endocrine neoplasia type 2 RET protooncogene database: repository of MEN2-associated RET sequence variation and reference for genotype/phenotype correlations. Human mutation 30:548-556.

Moore MW, Klein RD, Farinas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature 382:76-79.

Mulligan LM, Eng C, Attie T, Lyonnet S, Marsh DJ, Hyland VJ, Robinson BG, Frilling A, Verellen-Dumoulin C, Safar A, et al. (1994) Diverse phenotypes associated with exon 10 mutations of the RET proto-oncogene. Human molecular genetics 3:2163-2167.

Nagy N, Guyer RA, Hotta R, Zhang D, Newgreen DF, Halasy V, Kovacs T, Goldstein AM (2020) RET overactivation leads to concurrent Hirschsprung disease and intestinal ganglioneuromas. Development (Cambridge, England) 147.

Natarajan D, Marcos-Gutierrez C, Pachnis V, de Graaff E (2002) Requirement of

signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development (Cambridge, England) 129:5151-5160.

Nguyen TT, Scimeca JC, Filloux C, Peraldi P, Carpentier JL, Van Obberghen E (1993) Co-regulation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1, and the 90-kDa ribosomal S6 kinase in PC12 cells. Distinct effects of the neurotrophic factor, nerve growth factor, and the mitogenic factor, epidermal growth factor. The Journal of biological chemistry 268:9803-9810.

Nishiyama C, Uesaka T, Manabe T, Yonekura Y, Nagasawa T, Newgreen DF, Young HM, Enomoto H (2012) Trans-mesenteric neural crest cells are the principal source of the colonic enteric nervous system. Nature neuroscience 15:1211-1218.

Okamoto M, Yoshioka Y, Maeda K, Bito Y, Fukumoto T, Uesaka T, Enomoto H (2019) Mice conditionally expressing RET(C618F) mutation display C cell hyperplasia and hyperganglionosis of the enteric nervous system. Genesis 57:e23292.

Oppenheim RW, Houenou LJ, Parsadanian AS, Prevette D, Snider WD, Shen L (2000) Glial cell line-derived neurotrophic factor and developing mammalian

545 motoneurons: regulation of programmed cell death among motoneuron subtypes.
546 J Neurosci 20:5001-5011.
547 Qiu MS, Green SH (1991) NGF and EGF rapidly activate p21ras in PC12 cells by
548 distinct, convergent pathways involving tyrosine phosphorylation. Neuron
549 7:937-946.
550 Romeo G, Ronchetto P, Luo Y, Barone V, Seri M, Ceccherini I, Pasini B, Bocciardi R,
551 Lerone M, Kaariainen H, et al. (1994) Point mutations affecting the tyrosine
552 kinase domain of the RET proto-oncogene in Hirschsprung's disease. Nature
553 367:377-378.
554 Santoro M, Carlomagno F, Romano A, Bottaro DP, Dathan NA, Grieco M, Fusco A,
555 Vecchio G, Matoskova B, Kraus MH, et al. (1995) Activation of RET as a
556 dominant transforming gene by germline mutations of MEN2A and MEN2B.
557 Science 267:381-383.
558 Taketomi T, Yoshiga D, Taniguchi K, Kobayashi T, Nonami A, Kato R, Sasaki M,
559 Sasaki A, Ishibashi H, Moriyama M, Nakamura K, Nishimura J, Yoshimura A
560 (2005) Loss of mammalian Sprouty2 leads to enteric neuronal hyperplasia and
561 esophageal achalasia. Nature neuroscience 8:855-857.
562 Tilghman JM, Ling AY, Turner TN, Sosa MX, Krumm N, Chatterjee S, Kapoor A, Coe

563 BP, Nguyen KH, Gupta N, Gabriel S, Eichler EE, Berrios C, Chakravarti A
564 (2019) Molecular Genetic Anatomy and Risk Profile of Hirschsprung's Disease.
565 The New England journal of medicine 380:1421-1432.
566 Tomuschat C, Puri P (2015) RET gene is a major risk factor for Hirschsprung's disease:
567 a meta-analysis. Pediatric surgery international 31:701-710.
568 Traverse S, Gomez N, Paterson H, Marshall C, Cohen P (1992) Sustained activation of
569 the mitogen-activated protein (MAP) kinase cascade may be required for
570 differentiation of PC12 cells. Comparison of the effects of nerve growth factor
571 and epidermal growth factor. Biochem J 288 (Pt 2):351-355.
572 Uesaka T, Nagashimada M, Enomoto H (2013) GDNF signaling levels control
573 migration and neuronal differentiation of enteric ganglion precursors. J Neurosci
574 33:16372-16382.
575 Uesaka T, Nagashimada M, Yonemura S, Enomoto H (2008) Diminished Ret expression
576 compromises neuronal survival in the colon and causes intestinal aganglionosis
577 in mice. The Journal of clinical investigation 118:1890-1898.
578 Wagner SM, Zhu S, Nicolescu AC, Mulligan LM (2012) Molecular mechanisms of RET
579 receptor-mediated oncogenesis in multiple endocrine neoplasia 2. Clinics (Sao
580 Paulo) 67 Suppl 1:77-84.

581 Wells SA, Jr., Pacini F, Robinson BG, Santoro M (2013) Multiple endocrine neoplasia
582 type 2 and familial medullary thyroid carcinoma: an update. The Journal of
583 clinical endocrinology and metabolism 98:3149-3164.
584 Wolfe HJ, Melvin KE, Cervi-Skinner SJ, Saadi AA, Juliar JF, Jackson CE, Tashjian AH,
585 Jr. (1973) C-cell hyperplasia preceding medullary thyroid carcinoma. The New
586 England journal of medicine 289:437-441.
587 Wu JJ, Chen JX, Rothman TP, Gershon MD (1999) Inhibition of in vitro enteric
588 neuronal development by endothelin-3: mediation by endothelin B receptors.
589 Development (Cambridge, England) 126:1161-1173.
590 Yin L, Puliti A, Bonora E, Evangelisti C, Conti V, Tong WM, Medard JJ, Lavoue MF,
591 Forey N, Wang LC, Manie S, Morel G, Raccurt M, Wang ZQ, Romeo G (2007)
592 C620R mutation of the murine ret proto-oncogene: loss of function effect in
593 homozygotes and possible gain of function effect in heterozygotes. Int J Cancer
594 121:292-300.
595 Young HM, Hearn CJ, Farlie PG, Canty AJ, Thomas PQ, Newgreen DF (2001) GDNF is
596 a chemoattractant for enteric neural cells. Developmental biology 229:503-516.
597
598

599

600 **Figure legends**

601 **Figure 1**. *Ret*$^{51(C618F)/51(C618F)}$ mice display hypertrophy of enteric ganglia

602 A, Whole-mount Phox2b staining (green) of enteric neurons in the myenteric plexus of
603 the small intestine from P0 *Ret*$^{+/+}$, *Ret*$^{51/51}$, and *Ret*$^{51(C618F)/51(C618F)}$ mice. **B,**
604 Quantification of Phox2b^{+} enteric neuron numbers in the small intestine and the colon
605 from P0 *Ret*$^{+/+}$ (n=3), *Ret*$^{51/51}$ (n=3), and *Ret*$^{51(C618F)/51(C618F)}$ (n=6) mice. **p* = 0.001, ***p*
606 <0.0001, one-way ANOVA with Tukey's *post hoc* test. **C,D**, Detection of EdU
607 (magenta) incorporated into Phox2b^{+} ENS precursors (green) in the midgut from E12.5
608 (**C**) and E14.5 (**D**) *Ret*$^{51/51}$ and *Ret*$^{51(C618F)/51(C618F)}$ fetuses. The gut was labeled by a
609 two-hour pulse of EdU. The graphs (right panels) display the rate of ENS precursor
610 proliferation at E12.5 and 14.5 *Ret*$^{51/51}$ (n=3) and *Ret*$^{51(C618F)/51(C618F)}$ (n=3) fetuses. **p*
611 <0.05, unpaired t-test. Scale bars: **A,** 50 μm; **C, D,** 20 μm.

612

613 **Figure 2**. *Ret*$^{51(C618F)/51(C618F)}$ mice exhibit complete gut colonization by ENS precursors
614 Phox2b-labeled ENS precursors and neurons (green) in the developing gut in *Ret*$^{51/51}$
615 and *Ret*$^{51(C618F)/51(C618F)}$ mice at E12.5 (upper panels) and P0 (lower panels). Migration
616 of ENS precursors is slightly delayed at E12.5, but gut colonization by them is

617 completed at P0. Arrowheads depict the front of the migrating ENS precursors. Ce,
618 cecum; Co, colon; Si, small intestine; Hg, hindgut, Mg, midgut. Scale bars: 250 μm
619 (upper panels); 500 μm (lower panels).
620
621 **Figure 3**. *Ret*$^{51(C618F)/-}$ mice exhibit intestinal aganglionosis.
622 **A,B**, Whole-mount images of the enteric neurons stained with anti-PGP-9.5 (A) or
623 labeled by GFP (B) in P0 *Ret*$^{51/+}$ and *Ret*$^{51(C618F)/+}$, *Ret*$^{51/EGFP}$, and *Ret*$^{51(C618F)/EGFP}$ gut.
624 Complete gut colonization by ENS cells was seen in *Ret*$^{51/+}$, *Ret*$^{51(C618F)/+}$ and *Ret*$^{51/EGFP}$
625 mice (white arrowheads), while *Ret*$^{51(C618F)/EGFP}$ mice exhibited disrupted colonization
626 of the gut by ENCCs. The wavefront (yellow arrowheads) was defined as the most
627 caudal continuous strands of EGFP^{+} cells. Some *Ret*$^{51(C618F)/EGFP}$ mice show skip
628 segment aganglionosis where small regions of the colon contain enteric ganglia (white
629 dotted region). **C,** The proportion of three types of aganglionic phenotype (small
630 intestinal, skip segment and colonic aganglionosis). Ce, cecum; Co, colon; Si, small
631 intestine. Scale bars: **A-B,** 1 mm.
632
633 **Figure 4.** Detection of a few enteric neurons in the hindgut of *Ret*$^{51(C618F)/EGFP}$ embryos.
634 Whole mount preparation of embryonic gut showing the presence of a few

differentiating enteric neurons (A, inset) revealed by anti-PGP9.5 antibody (B). Scale bars: **A**, 100 µm; **B**, 50 µm.

Figure 5. Reduced RET51(C618) expression leads to premature differentiation of ENS precursors at the migratory wavefront.

A, Whole-mount images of GFP-labeled cells in the gut from E12.5 $Ret^{51/EGFP}$ and $Ret^{51(C618F)/EGFP}$ embryos (left panels). GFP^+ cells in the migratory wavefront were stained by anti-Sox10 (right panels), whereas Sox10-negative GFP^+ cells (white arrowheads) were found at the delayed migratory wavefront (open arrowhead) of $Ret^{51(C618F)/EGFP}$ gut. **B**, Whole-mount images of GFP-labeled cells stained with anti-PGP9.5 in E12.5 $Ret^{51/EGFP}$ and $Ret^{51(C618F)/EGFP}$ gut. PGP9.5-labeled GFP^+ cells were detected at the delayed migratory wavefront of $Ret^{51(C618F)/EGFP}$ gut. **C**, Immunohistochemical staining for GFP (green), Sox10 (blue) and activated ERK (pERK, magenta) in ENS cells of $Ret^{51/EGFP}$ and $Ret^{51(C618F)/EGFP}$ embryos at E12.5. In the migratory wavefront of $Ret^{51(C618F)/EGFP}$ embryos, pERK was mainly observed in GFP^+ and $Sox10^-$ differentiating neurons (white arrowheads). Hg, hindgut; Mg, midgut; Ro, rostal; Ca, caudal. Scale bars: **A,** 250 µm; **B,** 25 µm; **C,** 20 µm.

653

654 **Figure 6**. Reducing *Gdnf* gene dosage moderately rescues ENS phenotype of

655 $Ret^{51(C618F)/-}$mice.

656 **A,** Representative images of P0 $Ret^{51/EGFP}$/ $Gdnf^{+/-}$ and $Ret^{51(C618F)/EGFP}$/ $Gdnf^{+/-}$ large

657 intestine showing complete colonization with GFP-positive enteric neurons. **B,**

658 Comparison of ENS wavefront location between $Ret^{51(C618F)/EGFP}$ and $Ret^{51(C618F)/EGFP}$/

659 $Gdnf^{+/-}$ mice at P0. Reduction of *Gdnf* gene dosage significantly ameliorated the

660 severity of enteric aganglionosis (chi-square test, $p<0.01$). **C,** Representative images of

661 E12.5 $Ret^{51/EGFP}$/ $Gdnf^{+/-}$ and $Ret^{51(C618F)/EGFP}$/ $Gdnf^{+/-}$ gut displaying colonization by

662 GFP-positive ENS precursors. White arrowheads indicate the location of ENS precursor

663 wavefront. **D,** Whole-mount GFP, Sox10 and pERK pathway stainings of ENS cells of

664 $Ret^{51(C618F)/EGFP}$/ $Gdnf^{+/-}$ embryos at E12.5. Activation of ERK was not observed in ENS

665 precursors at the migratory wavefront. Ce, cecum; Co, colon; Si, small intestine; Hg,

666 hindgut; Mg, midgut. Scale bars: **A,** 1000 μm; **C,** 250 μm; **D,** 20 μm.

667

668 **Figure 7**. The severity of interruption of the ENS migration depends on the *Ednrb* gene

669 dosage in $RET^{51(C618F)/-}$ mice.

670 **A,** Whole_mount GFP staining of P0 $Ret^{51/EGFP}$/ $Ednrb^{+/-}$ and $Ret^{51(C618F)/EGFP}$/ $Ednrb^{+/-}$

671 gut. White arrowheads indicate the location of wavefront of enteric neurons. **B,**
672 Comparison of location of ENS wavefront between *Ret*$^{51(C618F)/EGFP}$ and *Ret*$^{51(C618F)/EGFP}$/
673 *Ednrb* $^{+/-}$ mice at P0. Reduction of *Ednrb* gene dosage increased the severity of enteric
674 aganglionosis of *Ret*$^{51(C618F)/EGFP}$ mice. **C,** Whole-mount GFP staining of the enteric
675 neurons in E12.5 *Ret*$^{51(C618F)/EGFP}$/ *Ednrb*$^{+/-}$ gut. White arrowhead indicates location of
676 the wavefront of ENS precursors. **D,** Whole-mount GFP, PGP9.5, Sox10 and pERK
677 pathway stainings of ENS cells of *Ret*$^{51(C618F)/EGFP}$ / *Ednrb*$^{+/-}$ embryos at E12.5.
678 Activation of ERK was observed in ENS precursors at the migratory wavefront. **E,**
679 Immunohistochemical detection of GFRα1 (green) in ENS progenitors (whose nuclei
680 marked in magenta by anti-Phox2b antibody) of wild type, *Gdnf*$^{+/-}$ and *Ednrb*$^{+/-}$
681 embryos (E12.5). Ce, cecum; Co, colon; Si, small intestine; Hg, hindgut; Mg, midgut.
682 Scale bars: **A,** 1 mm; **C,** 250 μm; **D,** 20 μm.