Increased RET Activity Coupled with a Reduction in the RET Gene Dosage Causes Intestinal Aganglionosis in Mice

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Abstract

Mutations of the gene encoding the RET tyrosine kinase causes Hirschsprung’s 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 Ret51(C618F)/51(C618F) mice displayed hyperganglionosis of the ENS, Ret51(C618F)/- mice exhibited severe intestinal aganglionosis because of premature neuronal differentiation. Reduced levels of glial cell-derived neurotrophic factor (GDNF), a RET-activating neurotrophic factor, ameliorated the ENS phenotype of Ret51(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.

Key words: cell signaling; enteric nervous system; Hirschsprung’s disease; neuronal differentiation; RET

Significance Statement

Mutations of the RET gene have been identified in Hirschsprung’s 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

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

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

Together, HSCR-associated and MTC-associated RET mutations display distinct features, and, although the whole spectrum of biological effects by those RET mutations have not been fully elucidated, current consensus holds that HSCR is caused by inactivating mutations of the RET gene whereas MTC is induced by activating mutations of RET (Hansford and Mulligan, 2000).

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

Materials and Methods

Mice

The generation and characterization of Ret51 and Ret51(C618F) mice have been described previously (Okamoto et al., 2019). We obtained RetGFp (a kind gift from J. Milbrandt, Washington University School of Medicine; Jain et al., 2006), Gdnffluorescent mice (a kind gift from V. Pachnis The Francis Crick Institute, London, UK; Moore et al., 1996), and Ednrbflex3 mice (a kind gift from M. L. Epstein, University of Wisconsin-Madison; Druckenbrod et al., 2008). Ednrbfluorescent mice were obtained by crossing Ednrbflex3 mice to Actb::Cre mice (stock #019099; The Jackson Laboratory; 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 postnatal day (P)0 pups were fixed with 4% paraformaldehyde (PFA) in PBS containing 10 mM phosphate buffer, pH 7.4, 137 mM sodium chloride, and 2.7 mM potassium chloride overnight at 4°C and incubated in 1% Triton X-100 in PBS for 30 min at room temperature. After fixation and permeabilization, the preparations were incubated in 0.1 M glycine in PBS for 2–6 h and processed for immunohistochemistry. For the preparations from P0 pups, blocking solution contains 5% skim milk, 5% DMSO, 1% Tween 20 in PBS. The following antibodies were used: guinea pig anti-Phox2b (1:1000, home-made, raised against the C-terminal region of Phox2b (RRID:AB_2313690; Pattyn et al., 1997), goat anti-Sox10 (1:300, catalog #sc-17342, Santa Cruz Biotechnology Inc., RRID: AB_2195374), rabbit anti-
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). Ret51(C618F)/51(C618F) mice were born apparently normally at an expected Mendelian ratio but all died of unknown causes within 24 h after birth (Okamoto et al., 2019). In newborn (P0) Ret51(C618F)/51(C618F) mice, histologic analysis of the gut revealed that the density of enteric neurons in the myenteric layer of the small intestine appeared higher in Ret51(C618F)/51(C618F) mice than Ret51/51 mice (control; Fig. 1A). Neuronal count confirmed a significant increase in the numbers of myenteric neurons in both small intestine and colon of Ret51(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 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 Ret51(C618F)/51(C618F) embryos as compared with Ret51/51 embryos in both of these developmental periods [Ret51(C618F)/51(C618F) vs Ret51/51 in the midgut; 43.3 ± 2.9% vs 31.7 ± 6.6% (p = 0.019) at E12.5 and 13.9 ± 2.1% vs 10.2 ± 0.9% (p = 0.046) at E14.5, respectively; Fig. 1C,D]. Thus, the increase in enteric neuron numbers in newborn Ret51(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 therefore examined the migration of ENS precursors in Ret51(C618F)/51(C618F) embryos. Unexpectedly, the migratory wavefront of ENS precursors was always slightly delayed in Ret51(C618F)/51(C618F) embryos as compared with control embryos at E12.5 (Fig. 2, upper panel). However, this delay was only transient and compensated for before birth. The ENS was fully developed in all of Ret51(C618F)/51(C618F) neonates (Fig. 2, lower panel).

In adult Ret51(C618F)/+ mice, we detected focal hyperplasia of thyroid C cells (Okamoto et al., 2019), a precancerous condition that leads to MTC (Wolfe et al., 1973).
Together, these results indicate that, consistent with its enhanced activity in vitro, RET51(C618F) confers gain-of-function effects on development of the ENS and thyroid C cells.

**Ret51(C618F)/-** mice display intestinal aganglionosis

We moved on to examine the effects of a reduction in the dosage of the RET gene because reduced RET expression is known to confer susceptibility to intestinal aganglionosis in both human and mice (Emison et al., 2005; Uesaka et al., 2008). We crossed Ret51/51 or Ret51(C618F)/51(C618F) mice to RetEGFP/+ mice in which one of the Ret alleles was replaced by the Ret-EGFP allele (Ret null). Consistent with previous 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^51/51 mice (Fig. 3A, B, left). In 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 ~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, eight mice (20% of all examined) displayed skip segment-type aganglionosis (Fig. 3B, third picture). This skip segment appears to be developed at least partially because of 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

Figure 2. Ret^51(C618F)/51(C618F) mice exhibit complete gut colonization by ENS precursors. Phox2b-labeled ENS precursors and neurons (green) in the developing gut in Ret^51/51 and Ret^51(C618F)/51(C618F) mice at E12.5 (upper panels) and P0 (lower panels). Migration of ENS precursors is slightly delayed at E12.5, but gut colonization by them is completed at P0. Arrowheads depict the front of the migrating ENS precursors. Hg, hindgut; Mg, midgut. Scale bars: 250 μm (upper panels) and 500 μm (lower panels).

Figure 3. Ret^51(C618F)/− mice exhibit intestinal aganglionosis. A, B, Whole-mount images of the enteric neurons stained with anti-PGP-9.5 (A) or labeled by GFP (B) in P0 Ret^51/+, Ret^51(C618F)/+, Ret^51/EGFP, and Ret^51(C618F)/EGFP gut. Complete gut colonization by ENS cells was seen in Ret^51/+, Ret^51(C618F)/+, and Ret^51/EGFP mice (white arrowheads), while Ret^51(C618F)/EGFP mouse exhibited disrupted colonization of the gut by ENCCs. The wavefront (yellow arrowheads) was defined as the most caudal continuous strands of EGFP⁺ cells. Some Ret^51(C618F)/EGFP mice show skip segment aganglionosis where small regions of the colon contain enteric ganglia (white dotted region). C, The proportion of three types of aganglionic phenotype (small intestinal, skip segment, and colonic aganglionosis). Ce, cecum; Co, colon; Si, small intestine. Scale bars: 1 mm (A, B).
E13.5, a period 2 d 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 Ret51(C618F)/EGFP embryos

To investigate the mechanism underlying the impaired ENS development in Ret51(C618F)/EGFP mice, we conducted whole-mount immunohistochemical analyses of embryonic gut (E12.5). In control (Ret51/EGFP) embryos, ENS progenitors at the migrating wavefront invaded the proximal colon and expressed both RET (revealed by GFP fluorescence) and Sox10 (Fig. 5A, left), indicating those cells are immature progenitors. Consistent with this expression pattern, none of the cells at the wavefront expressed PGP9.5 (Fig. 5B, left), a marker for neuronal differentiation. In contrast, in Ret51(C618F)/EGFP embryos, Sox10 expression was lost in many cells at the wavefront (Fig. 5A, right, arrowheads). Associated with this change, we found aberrant expression of PGP9.5 in ENS progenitors at the migratory wavefront, which was located primarily in the midgut (Fig. 5B, right). These results indicate that premature neuronal differentiation is induced in ENS progenitors at the migratory wavefront in Ret51(C618F)/EGFP embryos.

A previous study revealed that elevation of ERK activity is associated with induction of neuronal differentiation in ENS progenitors (Uesaka et al., 2013). We examined ERK activation by whole-mount staining of embryonic gut (E12.5) using anti-phospho-Erk (pErk) antibodies. In Ret51/EGFP embryos, pErk-positive ENS progenitors were abundant in proximal regions of the midgut, whereas such cells were almost undetectable at the wavefront region (Fig. 5C, right upper panels). In contrast, pErk-positive cells were frequently observed not only in the proximal midgut but also in the wavefront regions in Ret51(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 Ret51(C618F) mice, we examined whether severity of the phenotype changes in Ret51(C618F)/EGFP mice on Gdnf+/− background. By whole-mount GFP staining of the neonatal gut (n = 20), we found

Figure 4. Detection of a few enteric neurons in the hindgut of Ret51(C618F)/EGFP embryos. 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: 100 μm (A) and 50 μm (B).
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 Ret51/EGFP and Ret51(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 of Ret51(C618F)/EGFP gut. **B**, Whole-mount images of GFP-labeled cells stained with anti-PGP9.5 in E12.5 Ret51/EGFP and Ret51(C618F)/EGFP gut. PGP9.5-labeled GFP+ cells were detected at the delayed migratory wavefront of Ret51(C618F)/EGFP gut. **C**, Immunohistochemical staining for GFP (green), Sox10 (blue), and activated ERK (pERK, magenta) in ENS cells of Ret51/EGFP and Ret51(C618F)/EGFP embryos at E12.5. In the migratory wavefront of Ret51/EGFP.
that most of Ret<sup>51(C618F)/EGFP</sup>/Gdnf<sup>+/−</sup> mice (80%) displayed colonic aganglionosis (Fig. 6A), which stood in a sharp contrast to the ENS phenotype of Ret<sup>51(C618F)/EGFP</sup> that showed mostly extensive aganglionosis (aganglionic segment exceeding to the small intestine). χ<sup>2</sup> test of independence confirmed the significant differences between Ret<sup>51(C618F)/EGFP</sup>/Gdnf<sup>+/−</sup> and Ret<sup>51(C618F)/EGFP</sup>/Gdnf<sup>+/−</sup> mice (p = 0.005 < 0.01). Interestingly, in one case, the ENS was found fully developed up to the anal end (Fig. 6B). Moreover, skip segment-type aganglionosis, which was identified in 24% of Ret<sup>51(C618F)/-</sup> mice, was not detected in any of Ret<sup>51(C618F)/EGFP</sup>/Gdnf<sup>+/−</sup> mice. These results collectively indicate that reduction in GDNF levels exerts significant rescue effects on severe aganglionosis phenotype (Fig. 6A,B).

At E12.5, migration of ENS precursors was delayed in Ret<sup>51(C618F)/EGFP/Gdnf</sup>−/− embryos as compared with Ret<sup>51/EGFP/Gdnf</sup>−/− embryos (Fig. 6C). To examine the effect of the reduction of Gdnf gene dosage on intracellular signaling, whole-mount pERK staining was performed on embryos, pERK was mainly observed in GFP<sup>+</sup> and Sox10<sup>−</sup> differentiating neurons (white arrowheads). Hg, hindgut; Mg, midgut. Scale bars: 250 μm (A, left panel), 50 μm (A, right panel), 25 μm (B), and 20 μm (C).

Figure 6. Reducing Gdnf gene dosage moderately rescues ENS phenotype of Ret<sup>51(C618F)/</sup> mice. A, Representative images of P0 Ret<sup>51/EGFP/Gdnf</sup>−/− and Ret<sup>51(C618F)/EGFP</sup>/Gdnf<sup>+/−</sup> large intestine showing complete colonization with GFP-positive enteric neurons. B, Comparison of ENS wavefront location between Ret<sup>51(C618F)/EGFP/Gdnf</sup>−/− and Ret<sup>51/C618F)/EGFP/Gdnf</sup>−/− mice at P0. Reduction of Gdnf gene dosage significantly ameliorated the severity of enteric aganglionosis (χ<sup>2</sup> test, p < 0.01). C, Representative images of E12.5 Ret<sup>51/EGFP/Gdnf</sup>−/− and Ret<sup>51(C618F)/EGFP/Gdnf</sup>−/− gut displaying colonization by GFP-positive ENS precursors. White arrowheads indicate the location of ENS precursor wavefront. D, Whole-mount GFP, Sox10, and pERK pathway stainings of ENS cells of Ret<sup>51(C618F)/EGFP/Gdnf</sup>−/− embryos at E12.5. Activation of ERK was not observed in ENS precursors at the migratory wavefront. Ce, cecum; Co, colon; Si, small intestine; Hg, hindgut; Mg, midgut. Scale bars: 1000 μm (A), 250 μm (C), and 20 μm (D).
the gut of Ret51(C618F)/Ednrb+/–/Gdnf+/– embryos (E12.5). Similar to wild-type or Ret51/EGFP embryos (Fig. 4C, top), ERK phosphorylation was undetectable at the wavefront regions of Ret51(C618F)/EGFP/Gdnf+/–/– embryos (Fig. 6D, right). These data reveal that GDNF-mediated activation of RET51(C618F) is responsible for aberrant phosphorylation of ERK in ENS precursors at the wavefront and causes intestinal aganglionosis in Ret51(C618F)/– embryos.

An allelic loss of the Ednrb gene exacerbates the ENS phenotype of Ret51(C618F)/–/– mice

Previous studies revealed a genetic interaction between the Ret and Ednrb genes in HSCR pathogenesis. Either Ret heterozygosity or the Ednrb Is/Is allele alone exerts no adverse effect on ENS development, but induces severe intestinal aganglionosis when combined (Carrasquillo et al., 2002). Ednrb signaling regulates multiple processes of ENS development including migration, proliferation and differentiation of ENS precursors (Barlow et al., 2003; Kruger et al., 2003). We examined a potential genetic interaction between the Ret51(C618F) allele and the Ednrb gene by crossing Ret51(C618F)/–/– mice to Ednrb+/+/–/– mice. These mice displayed no adverse effect on ENS development, but induced severe intestinal aganglionosis in sharp contrast to that of Ret51(C618F)/–/– embryos (Fig. 7A–C). Immunohistochemical examination of ENS precursors revealed robust phosphorylation of ERK and loss of Sox10 expression at the wavefront (Fig. 7D). Reduction of one copy of the Ednrb gene leads to exacerbation of the aganglionosis phenotype, which contrasted the ameliorating effect by the reduction of the Gdnf gene dosage. This difference is not caused by the differential expression levels of GFRα1, the cognate receptor for GDNF involved in HSCR pathogenesis (Lui et al., 2002), as it was expressed at comparable levels in Gdnf+/–/–, Ednrb+/–/–, and wild-type embryos (Fig. 7E). At 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 because of intermolecular 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 homozygous fashion display kidney agenesis and intestinal aganglionosis, a phenotype identical to that of Ret-deficient mice (Carniti et al., 2008; Yin et al., 2007). RET(C618F) examined in this study exhibited distinct properties. Unlike other C618 or C620 mutants, RET(C618F) is expressed on the cell surface (Okamoto et al., 2019). Although phosphorylation levels of RET(C618F) are slightly higher than those of wild-type RET, GDNF stimulation further enhances phosphorylation of RET(C618F) (Okamoto et al., 2019). Thus, RET(C618F) is a GDNF-responsive RET-activating mutant. Consistent with these biochemical properties, Ret51(C618F)/51(C618F) mice had increased numbers of enteric neurons because of enhanced proliferation of ENS precursors. Surprisingly, despite the activating nature of RET51(C618F), Ret51(C618F)/–/– mice displayed severe intestinal aganglionosis, in sharp contrast to Ret51(C618F)/–/– mice (control), which exhibited no ENS deficit. This study provides evidence, for the first time to our knowledge, that RET-activating mutations can cause intestinal aganglionosis when coupled with a reduction in the Ret gene dosage.

It is important to note that, the RET C618F allele displays genetic interaction with the Ednrb gene, which is known as a modifier gene for HSCR carrying mutations in the RET gene. Our findings suggest a novel pathogenetic mechanisms of HSCR by 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 Ret51(C618F)/–/– mice displayed skip-segment aganglionosis. Ret51(C618F)/–/– mice thus serve as the first valuable platform to investigate the molecular and cellular mechanisms underlying this mysterious condition. Histologic examination of Ret51(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 Ret51(C618F)/–/– embryos stands in sharp contrast to that of Ret51(C618F)/–/– embryos, in which ENS precursors underwent proliferation rather than differentiation. Although the exact mechanism by which ENS precursors adopt 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 RET51(C618F)/RET51(C618F) and RET51(C618F)/RET51(C618F) embryos. We tried to examine this possibility by culturing ENS precursors and conducting biochemical analyses.
Unfortunately, however, mutant Ret51(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 Ret51(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, on 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-containing kinase efficiently. It is currently unknown how RET51(C618F) is localized and activates intracellular signaling molecules on the cell surface. In these respects, RET51(C618F) may not reflect enhanced activity of purely wild-type RET. RET51(C618F) has unique biochemical properties among MEN-associated RET mutants, which suggest that all of RET-activating mutations do not necessarily cause the intestinal aganglionosis by RET gene dosage reduction.

Amelioration of the phenotype in Ret51(C618F)−/− embryos by the Gdnf gene reduction indicates that GDNF-mediated activation of RET51(C618F) is responsible for the severe aganglionic phenotype. Although NRTN also activates RET in developing ENS (Heuckeroth et al., 1998), contribution of NRTN-mediated RET(C618F) activation to the aganglionic phenotype is unlikely because expression of GFRα2, the cognate receptor for NRTN, occurs later than a period when the aganglionic phenotype in Ret51(C618F)−/− embryos becomes obvious. It is important to note that, in normal ENS development, enteric neuron numbers are determined primarily by the levels of GDNF signaling. Mice heterozygous for the GDNF-deficient allele (Gdnf+/− mice) display reduced numbers of enteric neurons (Gianino et al., 2003). In contrast, reduction of Sprouty2, an inhibitor of Erk phosphorylation downstream of RET/GDNF signaling, leads to hyperganglionosis of the gut (Taketomi et al., 2005). This hyperganglionosis phenotype is suppressed on Gdnf+/− background. Evidence also suggests that RET 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 phospho-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.

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