Gfra1 Underexpression Causes Hirschsprung’s Disease and Associated Enterocolitis in Mice

L. Lauriina Porokuokka,1 Heikki T. Virtanen,1 Jere Lindén,2 Yulia Sidorova,1 Tatiana Danilova,1 Maria Lindahl,1 Mart Saarma,1,§ and Jaan-Olle Andressoo1,§

1Institute of Biotechnology, and 2Department of Veterinary Biosciences, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland

SUMMARY

A 70% to 80% underexpression of glial cell line-derived neurotrophic factor family receptor alpha-1 results in Hirschsprung’s disease and an associated enterocolitis phenotype in a new mouse model. Enterocolitis proceeds from goblet cell dysplasia, with mucin abnormalities, to epithelial damage. Reduced expression of glial cell line-derived neurotrophic factor family receptor a1 may contribute to Hirschsprung disease susceptibility.

BACKGROUND & AIMS: RET, the receptor for the glial cell line–derived neurotrophic factor (GDNF) family ligands, is the most frequently mutated gene in congenital aganglionic megacolon or Hirschsprung’s disease (HSCR). The leading cause of mortality in HSCR is HSCR-associated enterocolitis (HAEC), which is characterized by altered mucin composition, mucin retention, bacterial adhesion to enterocytes, and epithelial damage, although the order of these events is obscure. In mice, loss of GDNF signaling leads to a severely underdeveloped enteric nervous system and neonatally fatal kidney agenesis, thereby precluding the use of these mice for modeling postnatal HSCR and HAEC. Our aim was to generate a postnatally viable mouse model for HSCR/HAEC and analyze HAEC etiology.

METHODS: GDNF family receptor alpha-1 (GFRa1) hypomorphic mice were generated by placing a selectable marker gene in the sixth intron of the Gfra1 locus using gene targeting in mouse embryonic stem cells.

RESULTS: We report that 70%–80% reduction in GDNF co-receptor GFRa1 expression levels in mice results in HSCR and HAEC, leading to death within the first 25 postnatal days. These mice mirror the disease progression and histopathologic findings in children with untreated HSCR/HAEC.

CONCLUSIONS: In GFRa1 hypomorphic mice, HAEC proceeds from goblet cell dysplasia, with abnormal mucin production and retention, to epithelial damage. Microbial enterocyte adherence and tissue invasion are late events and therefore unlikely to be the primary cause of HAEC. These results suggest that goblet cells may be a potential target for preventative treatment and that reduced expression of GFRa1 may contribute to HSCR susceptibility. (Cell Mol Gastroenterol Hepatol 2019;7:655–678; https://doi.org/10.1016/j.jcmgh.2018.12.007)

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distention, and associated failure to thrive. The underlying pathogenic etiology is widely accepted to be based on defects in cranio-caudal migration, proliferation, differentiation, and survival of neuroblasts that originate from the neural crest in the early stage of pregnancy. The prevalence of HSCR is 1 in 5000 live births, and the disease generally is subdivided into short-segment and long-segment types based on the point at which histologically characterized aganglionosis begins. Currently, HSCR is treated by surgical removal of the aganglionic gut segment. The most life-threatening HSCR-associated complication is HSCR-associated enterocolitis (HAEC), which can occur preoperatively and/or postoperatively. HAEC develops in approximately 30% of patients with short-segment and 50% of patients with long-segment HSCR. The incidence of HAEC suggests either a genetic and/or environmental contribution, but the pathogenesis of HAEC remains poorly understood. Mucosal barrier dysfunction as a predisposing factor is one of the several proposed drivers for HAEC. HAEC is clinically characterized by fever, abdominal distention, diarrhea, and sepsis. Histopathologic features in the colon include crypt dilatation, mucus retention, enterocyte adherence of bacteria, a shift from acidic toward neutral mucin production, epithelial damage, leukocyte infiltration, ulceration, and, in the terminal stages, transmural necrosis and perforation. However, at least in part owing to a lack of suitable animal models, the order in which these HAEC-associated clinical features develop is currently poorly understood, which has hampered advances in the treatment and prevention of HAEC.

The most common genetic cause of HSCR is the presence of inactivating mutations in the receptor tyrosine kinase rearranged during transfection (RET), the signaling receptor for glial cell line–derived neurotrophic factor (GDNF); approximately 50% of cases are familial, and 15%–35% are sporadic cases. In addition, at least 13 other HSCR susceptibility genes have been identified, including GDNF, NRTN, EDNRB, EDN3, ECE1, SOX10, PHOX2B, KIF1Bβ, ZEB2, TCF4, and TTF1; together, mutations in these genes account for approximately 20% of all HSCR cases. HSCR penetrance also is known to be influenced by gene expression levels because copy number variants of MAPK10, ZFHX1B, and SOX2 loci were associated with HSCR in a pilot study on 67 candidate genes. The underlying genetic cause is unknown for approximately half of all sporadic cases of HSCR.

RET is activated upon binding with a complex comprising GDNF and GDNF family receptor alpha-1 (GFRα1), a glycosylphosphatidylinositol–anchored protein. Studies using transgenic mice have shed light on how GDNF signaling regulates the development of the enteric nervous system (ENS). In the developing mouse gut, ENS progenitor cells called enteric neural crest–derived cells (ENCCs) enter the foregut on embryonic day 9–9.5 (E9-E9.5). ENCCs migrate rostrocaudally along the gut toward a GDNF source, reach the proximal colon at E12, and colon colonization is complete by E14.5. In addition to regulating ENCC migration, GDNF signaling also controls the proliferation, differentiation, and survival of ENCCs. Mice that lack expression of genes encoding GDNF, GFRα1, or RET have a common phenotype that includes kidney agenesis and a lack of enteric ganglia distal to the stomach. However, heterozygous Gdnf, Gfra1, or Ret null-allele mice with a reduced gene dose have a relatively mild reduction in enteric neuron numbers and do not develop the clinical features of childhood HSCR or HAEC. Mouse studies using knock-in or timed conditional deletion alleles for Gfra1 or Ret support the importance of GFRα1/RET signaling in ENS development and survival through postnatal day 1 (P1). However, animal models with defective GDNF/GFRα1/RET signaling that phenocopy postnatal HSCR and/or HAEC are currently not available.

Mutations in the RET gene are the most common genetic cause of HSCR, however, causative mutations in Gfra1 have not been found. Here, we report the generation of Gfra1 hypomorphic mice with reduced expression of Gfra1 at the endogenous locus. These mice were generated by inserting a selectable marker gene within intron 6 of the Gfra1 gene. Homozygous Gfra1 hypomorphic (Gfra1<sup>hypo/hypo</sup>) mice have a 70%–80% reduction in Gfra1 expression in the developing gut and kidney. Although the kidneys develop normally, Gfra1<sup>hypo/hypo</sup> mice have congenital features reminiscent of childhood long-segment HSCR, with early postnatal onset of symptoms and accompanying progressive HAEC; these mice generally die at P7–P25. Our further analysis also suggests a possible explanation why reduced gene expression in heterozygous Gdnf, Gfra1, and Ret null-allele mice does not result in HSCR, whereas a more severe reduction in Gfra1 expression in Gfra1<sup>hypo/hypo</sup> mice does lead to HSCR. In addition, our analysis of Gfra1<sup>hypo/hypo</sup> mice has enabled us to better define the sequence of events in the pathogenesis of HAEC. Specifically, our results allow us to exclude bacterial enterocyte adherence as the primary cause of HAEC in our model, and suggest that goblet cell dysplasia is an early, ubiquitous event that precedes the epithelial changes, including crypt dilatation, mucus retention, and epithelial damage. Together, these findings suggest that goblet cells...
could be a candidate target for developing preventative therapies for HAE and that reduced expression of GFRα1 may represent a novel susceptibility trait for HSCR.

**Results**

*Generation and Primary Characterization of Gfра1hypo/hypo Mice*

Insertion of a marker gene into an intron of a target gene often interferes with expression of the target, yielding a hypomorphic allele. We inserted a pu tk cassette into intron 6 of the Gfра1 gene (Figure A-C) and analyzed the phenotype of homozygous (Gfра1pu,tk/pu,tk) mice generated by crossing 2 heterozygous animals. Compared with their expected Mendelian inheritance, homozygous mice were under-represented by approximately 3- to 4-fold at the time of weaning (P18-P25); moreover, homozygous mice developed severe abdominal swelling, failed to thrive, showed increasing inactivity, and other signs of discomfort. Of the 8 homozygous mice that were monitored beginning at P15, none survived beyond P27; because of ethical considerations, we did not proceed with subsequent survival studies. During further experiments, animals that showed obvious signs of discomfort, including abdominal swelling, cachexia, and inactivity, were euthanized. One or more of those signs usually became evident by P4, with gradual progression until the end stage/euthanasia, usually between P7 and P25. Necropsy performed at P18-P25 showed marked caudal gut distention (Figure 1D) starting between the distal ileum and the mid-colon. Four of 8 homozygous animals analyzed at P18-P25 had acute erythema/hyperemia in the gastrointestinal (GI) tract, indicative of acute inflammation (Figure 1D, right panel). Upon further inspection, narrowed, contracted gut that was distal to the distention was less flexible and stiff, a feature commonly reported for aganglionic gut segments in HSCR patients.

*Analysis of Gfра1, Ret, and Gdnf Expression*

Quantitative real-time polymerase chain reaction (PCR) analysis performed at E13.5 showed that Gfра1 messenger RNA (mRNA) levels were reduced by 75%-80% in the kidneys, stomach, and duodenum of Gfра1hypo/hypo mice; expression levels decreased further toward the colon, where Gfра1 mRNA levels were reduced by approximately 90% compared with control animals (Figure 1E). Ret mRNA levels were normal in the stomach and duodenum, but sharply decreased in the ileum of Gfра1hypo/hypo mice. In the cecum and colon, Ret mRNA was not detectable in our analysis (Figure 1E). This was expected because in the developing gut Ret is expressed exclusively in ENCCs, whereas Gfра1 is expressed both in ENCCs and in the surrounding mesenchyme. As our parallel analysis showed, ENCCs in Gfра1hypo/hypo mice were missing distal from the midileum (see later).

Gdnf mRNA levels were slightly—albeit significantly—increased throughout the GI tract, possibly reflecting mechanistic compensation for the reduced expression of GDNF receptors (Figure 1E). Western blot analysis showed that the amount of GFRα1 protein in the gut segment from the duodenum to the ileum of Gfра1hypo/hypo mice was reduced by 75%-80% at E13.5 (Figure 1F and G). In the cecum and colon, Gfра1 protein was not detectable in our analysis. Kidney development in Gfра1hypo/hypo mice was generally normal (Figure 1H and I). Given the hypomorphic Gfра1 mRNA and protein levels measured in these mice, we renamed the Gfра1pu,tk allele the Gfра1 hypomorphic (Gfра1hypo) allele.

*Homozygous Gfра1hypo/hypo Mice Have Impaired Neuronal Progenitor Colonization and Develop Aganglionicosis and Hypertrophic Fibers in the Colon*

Mice that lack Gdnf, Gfра1, or Ret expression lack enteric nerves distal to the stomach. Moreover, experiments in which Gfра1 or Ret expression was deleted under temporal control in the developing gut showed that GDNF/GFRα1/RET signaling regulates the migration, proliferation, differentiation, and survival of ENCCs, particularly in the distal gut, in a gene dose-dependent manner. Consistent with these findings, innervation of the stomach in Gfра1hypo/hypo mice appeared normal at E13.5, and the number of neuronal progenitors decreased gradually toward the distal gut (Figure 2A). At E13.5, Gfра1hypo/hypo embryos lacked neuronal progenitors from the midileum onward (Figure 2A). In the developing gut, Ret is expressed exclusively in ENCCs, whereas Gfра1 is expressed both in ENCCs and in the surrounding mesenchyme. As shown in Figure 2A, neuronal progenitors in the distal gut in Gfра1hypo/hypo embryos are missing, explaining the observed craniocaudal reduction in Gfра1 mRNA and the absence of Ret mRNA in the distal gut (Figure 1E). Similar innervation defects were observed at E15.5 (ie, 3-4 days before birth in mice) (Figure 2F).

Heterozygous Gfра1 null-allele (Gfра1wild type [WT]/knockout [KO]) mice, which had an approximate 50% reduction in GFRα1 expression, had reduced enteric neuron fiber density and defects in gut function measured ex vivo; however, they did not have reduced numbers of enteric neurons, and they did not develop symptoms of either HSCR or HAEC. Our finding that a 70%-80% reduction in Gfра1 expression caused long-segment HSCR suggests a threshold effect in GFRα1-mediated downstream signaling. In the developing gut, 2 forms of the Gfра1 protein are present—the membrane-associated glycosylphosphatidylinositol-anchored form and the extracellular soluble form; these 2 forms can function cooperatively in mediating GDNF signaling in ENCCs. Because the concentration of a solute decreases steeply as a function of distance from the source, signaling via the soluble form of GFRα1 is likely to be particularly sensitive to decreased expression. We analyzed the concentration dependence of soluble GFRα1 on downstream signaling in an MGB7 fibroblast model system that stably expressed both Ret and a luciferase gene that reflected the activity of mitogen-activated protein kinases, a downstream target of RET signaling. We stimulated these cells with GDNF together with increasing concentrations of
soluble GFRα1 and measured a sharp response in RET downstream signaling within a relatively narrow GFRα1 concentration range of 1.35–6.75 nmol/L (Figure 2B). These results provide a possible explanation for the phenotype differences observed between Gfra1<sup>WT/KO</sup> and Gfra1<sup>hypo/hypo</sup> mice. In Gfra1<sup>WT/KO</sup> mice, the GFRα1 levels were within the effective range, whereas GFRα1 levels fell below the effective range in Gfra1<sup>hypo/hypo</sup> mice.

Long-segment HSCR is defined as aganglionosis proximal to the splenic flexure. Another hallmark clinical feature of HSCR is the presence of extrinsic hypertrophic nerve fibers in the distal colon. At E18.5, Gfra1<sup>hypo/hypo</sup> mice had varying-length aganglionic segments proceeding from the midcolon (long-segment HSCR) or even from the distal ileum (total colonic aganglionosis), and they had hypertrophic nerve fibers in the distal colon (Figure 2C and D).
Because the presence of hypertrophic fibers in the colon of HSCR patients is diagnosed postnatally, we analyzed the colon in postnatal (P20) Gfra1hypo/hypo mice and found that the hypertrophic fibers still were present (Figure 2F).

Gfra1hypo/hypo mice are born with a normal appearance; however, they then develop symptoms that resemble HSCR, including constipation, distention of the gut, and a failure to thrive (Figure 1J and see later), with 100% penetrance by P10. Consistent with early mortality, the genotypes in the animal cohort were at the expected Mendelian ratios at E18.5, but not at P10 or P15–P23 (Table 1). Immunohistologic analysis of the ENS in Gfra1hypo/hypo mice at P10 using the pan-neuronal marker ubiquitin C-terminal hydrolase L1 showed normal ganglia in the duodenum, reduced ganglia size in the distal ileum, and absent or rudimentary ubiquitin C-terminal hydrolase L1-positive structures in the colon, reflecting hypertrophic fibers in the colon (Figure 3A and B). Similar results were obtained using the glial fibrillary acidic protein marker (Figure 3C and D). Collectively, results presented in Figures 2 and 3 are consistent with previous results on time deletion of GFRa1 and RET until E18.5,18,25 and suggest that reduced Gfra1 expression in Gfra1hypo/hypo mice causes impaired cranio-caudal colonization of the gut by enteric neuronal progenitors, with subsequent aganglionosis of the distal gut and the occurrence of hypertrophic nerve fibers in the distal colon.

Hypertrophic Fibers in the Colon of Gfra1hypo/hypo Mice Are Cholinergic

Hypertrophic nerve fibers in the distal colon of HSCR patients are known to be cholinergic.40,41 The reduced flexibility of the aganglionic gut segment in HSCR patients is believed to result from excess extrinsic excitatory cholinergic stimuli and a lack of intrinsic inhibitory nitricergic stimuli.42,43 To examine the properties of the myenteric plexus in Gfra1hypo/hypo mice, we stained for acetylcholinesterase. Our analysis showed that Gfra1hypo/hypo mice have normal cholinergic innervation in the duodenum and proximal ileum, reduced cholinergic innervation in the distal ileum, a lack of cholinergic innervation in the midcolen, and hypertrophic cholinergic fibers in the distal colon (Figure 4A and B). Next, we stained the myenteric plexus for reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase, a marker of nitric oxide synthase–positive nitricergic enteric neurons. Our analysis showed that Gfra1hypo/hypo mice have normal numbers of NADPH-diaphorase-positive neurons in the duodenum, but a complete lack of NADPH-diaphorase–positive cells in the distal colon (Figure 4C and D), reminiscent of the histopathologic findings in patients with HSCR.42,43

Histopathologic Characterization of HSCR/HAEC in Gfra1hypo/hypo Mice

Improving our understanding of the histopathologic changes that occur in HACE may eventually help in developing more effective treatments. We used the histopathologic grading system for HAEC developed by Teitelbaum et al1 to analyze Gfra1hypo/hypo mice. Teitelbaum et al1 scored the histopathologic findings in HAEC in patients as follows: 0, no abnormalities; I, crypt dilatation and mucin retention; II, cryptitis or 2 crypt abscesses per high-power field; III, more than 2 crypt abscesses per high-power field; IV, fibrinopulent debris and mucosal ulceration; and V, transmural necrosis or perforation. However, compared with patients, mice with HAEC had milder infiltration of inflammatory cells into the crypts (milder cryptitis and crypt abscesses),44 although the epithelial pathologies were similar (see later); therefore, we modified the grading system to primarily reflect epithelial pathology (see the Materials and Methods section). Another characteristic feature associated with HAEC is an overproduction of mucus and the retention of mucus in the colon, as well as a shift from producing acidic mucins to producing neutral mucins.5,7 Mucins are produced primarily by goblet cells and form a protective barrier preventing bacterial enterocyte adherence.45 Goblet cell hyperplasia46 and altered goblet cell function47 have been reported in HSCR.

Figure 1. [See previous page]. Generation of Gfra1 hypomorphic mice and analysis of Gfra1 expression. (A) The Gfra1 hypomorphic allele was generated by inserting a puΔtk cassette1 into intron 6 in the Gfra1 gene. (B) Representative Southern blot analysis of EcoRV-digested genomic DNA isolated from embryonic stem cells, confirming correct targeting of Gfra1 via homologous recombination (expected sizes: targeted, 7564 bp; WT, 10397 bp). (C) PCR-based genotyping of mice using the primers indicated in panel A by F and R (expected sizes: WT, 88 bp; Gfra1Δtk/Δtk, 100 bp). (D) Gfra1hypo/hypo mice develop megacolon, constipation, and abdominal distention, and they fail to thrive (note that the Gfra1hypo/hypo mouse is smaller than the WT mouse). The point of dilation is indicated by black arrows. At P18–P25, 50% of Gfra1hypo/hypo mice, sacrificed due to obvious discomfort due to abdominal swelling, have clearly visible acute erythema/hyperemia in the gastrointestinal tract (white arrows) indicative of enterocolitis; representative images are shown. (E) Relative Gfra1, Gdnf, and Ret mRNA levels at E13.5 in the indicated organs measured by qPCR (N = 4). (F and G) Western blot analysis of Gfra1 protein in the gut segment spanning from the duodenum to the ileum in E12.5 embryos. (F) Representative Western blot (samples obtained from E12.5 Gfra1 KO mice were included as a negative control); (G) normalized average Gfra1 intensity (N = 3 independent experiments). (H) At P10, the kidneys in Gfra1hypo/hypo mice appear anatomically normal. (I) Hypertrophic Fibers in the Colon of Gfra1hypo/hypo mice are reflected in an overall failure of the mice to thrive. (J) Compared with WT littermates, kidney histology is grossly normal in P18 Gfra1hypo/hypo mice. F, forward primer; R, reverse primer; EV, EcoRV site; ex 6, exon 6; puΔtk/puΔtk, Gfra1Δtk/Δtk, KO/KO, Gfra1hypo/hypo, CHO, Chinese hamster ovary cells transfected with a plasmid encoding GFP (−) or Gfra1 (+). All summary data are represented as means ± SEM. *P < .05, **P < .01, and ***P < .001. Statistical analyses were performed using the Student t test, unless indicated otherwise.
Histopathologic characterization of HSCR/HAEC in E18.5, P4, and P10 Gfra1hypo/hypo mice. Histologic analysis of the colon in E18.5 Gfra1hypo/hypo mice showed no overt changes compared with control mice (Figure 5A and B). However, at P4, histologic analysis using Alcian blue (AB), periodic acid–Schiff (PAS), H&E, and Gram staining showed minimal to moderate epithelial damage accompanied by crypt dilatation and a mucin shift from acidic to neutral with mucus retention in the goblet cells (Figure 5C–F, Table 2). At P10 the differences were more advanced: slight to mild multifocal to diffuse epithelial hyperplasia, as well as dysplasia (ie, disorganization) accompanied by degeneration of surface colonocytes in Gfra1hypo/hypo mice (Figures 6A and B) was observed, indicating mild surface epithelial damage. In the hyperplastic and degenerated areas of the colon, the epithelial cells grew in disorganized, undulating rows with partly overlapping nuclei (Figure 6B and C). In addition, the crypts in the colon were mildly to moderately dilated with various degrees of mucin retention (Figure 6B and C), indicating an overproduction and retention of mucus in the colon. We also observed increased numbers of intraepithelial apoptotic cell remnants in 5 of 8 Gfra1hypo/hypo mice, indicating a degenerative process (Figure 6B). Importantly, the severity of these features varied between animals (the histopathologic data from each animal are presented in Table 3).

At P10, goblet cell pathology was more advanced, with a further shift in mucin production from acidic to neutral now also accompanied by goblet cell hyperplasia in all mutant mice. Notably, the earlier-described goblet cell pathology appeared in all mice regardless of the extent of the aforementioned epithelial pathology (Figure 6C, Table 3). More specifically, we observed that PAS-positive goblet cells, which are normally found in the apical and central parts of the crypts, were present at the base of the crypts in the Gfra1hypo/hypo colon (Figure 6B and C). On the other hand, AB, which stains acidic mucins, showed that AB-positive goblet cells, which are normally found at the base of crypts, are either absent or filled with a foamy mixture of both neutral and acidic mucins in the Gfra1hypo/hypo colon (Figure 6B and C). To gain insight into whether altered goblet cell physiology in the Gfra1hypo/hypo colon results from a deficit in the ENS in the colon and distal intestine, or from a reduction in GFRα1 levels in fetal mesenchyme in the whole intestine, we also analyzed goblet cells in the duodenum at P10 using AB-PAS staining. The number of goblet cells and the mucin profile were unchanged in the duodenum at P10 (Figure 6F–I).

Finally, both Gram and H&E staining showed no evidence of bacterial enterocyte adherence or microbial infection in the Gfra1hypo/hypo colon at P10 (Table 3). Based on our histopathologic grading system of HAEC, 3 of the 8 Gfra1hypo/hypo mice at P10 had a grade III HAEC score, 4 mice had a grade II HAEC score, and 1 mouse had a grade I HAEC score.

Taken together, these results suggest that a mucin shift from acidic to neutral in goblet cells at P4 is further

### Table 1. Distribution of Offspring Genotypes Born From Breedings Between Gfra1hwt/hypo Animals

| Distribution, number of animals (% total) | Expected, number of animals (% total) |
|------------------------------------------|--------------------------------------|
| E18.5         | WT        | 27 (25.23) | 26.75 (25) |
|              | WT/hypo   | 59 (55.14) | 53.5 (50)  |
|              | Hypo/hypo | 21 (19.63) | 26.75 (25) |
| P = .308     | WT/hypo   | 39 (24.38) | 37.5 (25)  |
|              | Hypo/hypo | 15 (18.75) | 37.5 (25)  |
| P = .018     | WT/hypo   | 96 (60.00) | 71.5 (50)  |
|              | Hypo/hypo | 25 (15.63) | 37.5 (25)  |
| P < .001     | WT/hypo   | 60 (57.14) | 52.5 (50)  |
|              | Hypo/hypo | 8 (7.62)   | 26.25 (25) |

**NOTE.** At E18.5, Gfra1hypo/hypo mice are present at the expected Mendelian ratio, but not at P10 (P < .05) or at P15-P23 (P < .001), chi-square analysis.

**Figure 2.** (See previous page). Impaired enteric neuronal progenitor colonization and hypertrophic nerve fibers in the distal gut of Gfra1 hypomorphic mice. (A) Immunohistochemistry using the pan-neuronal marker ubiquitin C-terminal hydrolase L1 in E13.5 WT and Gfra1hypo/hypo mice whole-mount GI tract preparations. Note the reduced numbers of neuronal progenitors (white arrows) beginning at the midjejunum of Gfra1hypo/hypo mice and the absence of neuronal progenitors from the distal and GDNF ileum onward; representative images are shown (N = 3–4 animals per genotype). (B) Dose-dependent activation of mitogen-activated protein kinase signaling by soluble GFRα1. MG87 fibroblasts stably expressing RET and the PathDetect Etk-1 luciferase system were treated with the indicated concentration of soluble GFRα1 (N = 4 biological replicates per experiment; N = 2 experiments). (C and D) Ubiquitin C-terminal hydrolase L1 immunohistochemistry was performed in E18.5 WT and Gfra1hypo/hypo mice from whole-mount GI tract preparations, showing reduced and scattered innervation in the midileum, an absence of innervation in the proximal colon, and hypertrophic fibers in the distal colon (white arrowheads, a hallmark feature of HSCR); the white arrows indicate neuronal somas. Representative images are shown (N = 3–4 animals per genotype). (E) Ubiquitin C-terminal hydrolase L1 immunohistochemistry performed on P20 whole-mount myenteric plexus preparations showing normal innervation in the duodenum, an absence of neuronal cell bodies in the colon, and hypertrophic fibers in the colon of Gfra1hypo/hypo mice; representative images are shown (N = 2–3 animals per genotype). (F) The ENCC colonization of the mouse hindgut is complete by E14.5. Representative image of sagittal gut sections immunostained with the pan-neuronal marker ubiquitin C-terminal hydrolase L1 in E15.5 WT and Gfra1hypo/hypo mice, showing that the enteric neuronal progenitors have reached the distal colon in the WT mice (white arrowheads); in contrast, enteric neuronal progenitors reached the proximal ileum (white arrowheads) in E15.5 Gfra1hypo/hypo mice, but are absent from the distal ileum, cecum, and colon. The point at which aganglionosis begins is indicated by a black arrowhead; representative images from WT and Gfra1hypo/hypo mice are shown. dist, distal; duo, duodenum; ile, ileum; jej, jejunum; prox, proximal.
enhanced and accompanied with hyperplasia at P10 and that those are generally ubiquitous early events in Gfra1hypo/hypo mice. On the other hand, the interanimal variations in epithelial damage, epithelial degeneration, crypt dilatation, and mucin retention (Table 3) suggest that these changes may be secondary to goblet cell dysfunction and appear later in HAEC.

Histopathologic characterization of HSCR/HAEC in P18–P25 Gfra1hypo/hypo mice. To analyze the features of advanced HAEC in our Gfra1hypo/hypo mice, we examined...
P18–P25 mice that showed obvious signs of discomfort, including cachexia, abdominal swelling, and reduced spontaneous activity. In these mice, we observed histopathologic features that are reminiscent of advanced HAEC in HSCR patients\(^5\)–\(^7\) (Figures 7 and 8; Table 4). Compared with P10 mice, in all animals analyzed the P18–P25 mice had more advanced levels of leukocyte accumulation, epithelial damage (including the presence of apoptotic cell remnants), mucus accumulation, and crypt dilatation–associated edema (Figures 6–8; Tables 3 and 4). The shift from acidic to neutral mucin production observed at P10 also clearly was evident at P18–P25 (Figure 7). Moreover, goblet cell hyperplasia (Figure 7B and C) was present only in mice with moderate epithelial damage; in contrast, mice with marked epithelial damage had either degenerated goblet cells or a combined phenotype of goblet cell hyperplasia and goblet cell degeneration (Figures 7C and 8B; Table 4). A variety of other features that are typical in advanced HAEC, including ulceration, necrotic purulent colitis, crypt abscesses, and/or the presence of focal bacterial aggregates, were observed in

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**Figure 4.** The hypertrophic nerve fibers in the distal gut of GFRα1 hypomorphic mice are cholinergic. (A) Acetylcholinesterase histochemistry at P10 showing similar innervation in the duodenum, as well as cholinergic hypertrophic nerve fibers characteristic of HSCR in the colon of Gfrα1\(^{hypo/hypo}\) mice. In the ileum, an image distal of the point of dilatation is shown (N = 3 animals per genotype). (B) Quantification of the acetylcholinesterase-positive plexus area in P10 WT and Gfrα1\(^{hypo/hypo}\) mice (N = 3 animals per genotype, ***P < .001). (C) NADPH diaphorase histochemistry for nitric oxide synthase (NOS)-positive neurons in P10 mice showing similar innervation in the duodenum and an absence of NOS-positive innervation in the colon of Gfrα1\(^{hypo/hypo}\) mice; N = 4–5 animals per genotype (N = 2 animals per genotype for the colon). (D) Quantification of NOS-positive plexus area in P10 WT and Gfrα1\(^{hypo/hypo}\) mice; N = 4–5 animals per genotype (N = 2 animals per genotype for the colon). Analysis of variance and the Tukey multiple comparisons tests were used for statistical analysis. n.a., not applicable for Gfrα1\(^{hypo/hypo}\) mice. ACE, acetylcholinesterase; dist, distal; prox, proximal (data are presented as means ± SEM). All summary data are presented as means ± SEM. *P < .05, **P < .01, and ***P < .001.
Figure 5. Histopathology of colon at E18.5 and P4. (A and B) Representative H&E-stained coronal sections of the colon at E18.5. No gross difference was observed between genotypes (N = 3–4 animals per genotype). (C and D). H&E staining from WT and Gfra1<sup>hypo/hypo</sup> colon at P4. Gfra1<sup>hypo/hypo</sup> animals typically show mild crypt dilatation accompanied with mucus retention (D2, black bold arrow). AB-PAS staining shows more acidic mucins in WT animals at the basal crypts (C3, open arrowhead) and more neutral mucins in Gfra1<sup>hypo/hypo</sup> animals (white arrowheads) quantified in F. **P < .01 (N = 3–4). (E) The number of goblet cells (C3 and D3, white arrows) was not changed at P4 (N = 4–5). Analysis of variance and the Tukey multiple comparisons test, data are presented as means ± SEM. All summary data are presented as means ± SEM.
Table 2. Summary of Histopathologic Findings at P4

| P4 ID   | Weight, g | Sex | Applied Teitelbaum et al grade | Surface epithelial injury | Crypt dilation | Intraepithelial apoptotic remnants | Goblet cell hyperplasia | Mucin shift to PAS | Basal Surface | Mucus retention | Bacteria in crypts | Enterocyte adherence | Granulocytes | Lymphocytes |
|---------|-----------|-----|--------------------------------|---------------------------|---------------|----------------------------------|-------------------------|-------------------|---------------|----------------|------------------|----------------------|---------------|-------------|
| WT 7615-2 | 3.02 F | - | 0 | - | - | No | No | AB | PAS | - | - | No | - | - |
| WT 7640-3 | 2.96 F | - | 0 | - | - | No | No | AB | PAS | - | - | No | - | - |
| WT 7640-4 | 3.16 M | 0 | - | - | - | No | No | AB | PAS | - | - | No | - | - |
| WT 7640-4a | 2.92 M | 0 | - | - | - | No | No | AB | PAS | - | - | No | - | - |
| WT/hypo 7615-4 | 3.15 F | 0 | - | - | - | No | No | AB | PAS | - | - | No | - | - |
| WT/hypo 7615-1 | 3.21 F | 0 | - | - | - | No | No | AB | PAS | - | - | No | - | - |
| WT/hypo 7615-5 | 3.06 M | 0 | - | - | - | No | No | AB | PAS | - | - | No | - | - |
| WT/hypo 7640-5a | 2.78 M | 0 | - | - | - | No | No | AB | PAS | - | - | No | - | - |
| Hypo/hypo 7615-3 | 2.60 F | I | Mild | ++ | - | No | Yes | PAS | PAS | ++ | - | No | - | - |
| Hypo/hypo 7640-1 | 2.38 F | I | Mild | ++ | - | No | Yes | PAS | PAS | ++ | - | No | - | - |
| Hypo/hypo 7640-1a | 2.34 M | I | Mild | +++ | - | No | Yes | PAS | PAS | ++ | - | No | - | - |
| Hypo/hypo 7640-3a | 2.44 M | I | Minimal | + | - | No | Yes | PAS | PAS | ++ | - | No | - | - |
| Hypo/hypo 7640-2 | 2.51 M | II | Moderate | ++ | + | No | Yes | PAS | PAS | ++ | - | No | - | - |
| Hypo/hypo 7640-3a | 2.15 M | I | Minimal | + | - | No | Yes | PAS | PAS | ++ | - | No | - | - |

**NOTE.** See the Materials and Methods section for details regarding the histopathologic grading.
Histopathology of colon P10

A

|          | 1          | 2          | 3          |
|----------|------------|------------|------------|
| HE       | WT         | HE         | AB-PAS     |

B

|          | 1          | 2          | 3          |
|----------|------------|------------|------------|
| HE       | Gfra1 hypo/hypo | HE       | AB-PAS     |

C

|          | 1          | 2          | 3          |
|----------|------------|------------|------------|
| HE       | AB-PAS     | Gfra1 hypo/hypo |          |

D

|          | Proximal col | Distal col |
|----------|--------------|------------|
| WT       | ***          | ***        |
| WT/hypo  | ***          | ***        |
| hyp/o hypo| ***          | ***        |

E

|          | % AB+ cells |
|----------|-------------|
| WT       | ***         |
| WT/hypo  | ***         |
| hyp/o hypo| ***        |

F

|          | WT         | Gfra1 hypo/hypo |
|----------|------------|-----------------|
| AB-PAS   | Duodenum   |                 |

G

|          | Goblet cells / villus |
|----------|-----------------------|
| WT       |                       |
| WT/hypo  |                       |
| hyp/o hypo|                      |

H

|          | % PAS+ cells/villus normalized to WT |
|----------|--------------------------------------|
| WT       |                                     |
| WT/hypo  |                                     |
| hyp/o hypo|                                     |
3 of the 4 animals investigated (Figure 7D and E). Bacterial enterocyte adherence on the other hand was observed in only 2 of the 3 mice with severe epithelial damage (Table 4); these same 2 mice also had bacteria within the colon crypts and blood vessels (Figures 7D and E and 88 and C), indicating sepsis. The histopathologic findings of each mouse examined at P4, P10, or P18–P25 are summarized in Tables 2, 3, and 4, respectively.

Taken together, these data suggest that Gfra1hypo/hypo animals recapitulate both the early and advanced histopathologic findings present in patients with HAEC.4,5,7

**Characterization of Cytokine mRNA Expression and Serum Protein Levels in Gfra1hypo/hypo Mice**

A mouse long-segment HSCR/HAEC model may allow identification of diagnostic markers and further explore the relationship between immune response and HAEC. As a first step in this direction we analyzed the expression of mRNAs for Il1a, Il1b, Il11ra, Il2, Il4, Il5, Il6, Il10, Il13, Il23, Tgfb1, Tnf, and Ifng selected based on their presumed or hypothetical involvement in GI tract inflammation. First, we performed a pilot study on mRNA expression in the colon at P10, when the HSCR/HAEC phenotype is readily detectable but less variable than at the end stage of the disease. Of 13 selected mRNAs, 7 were detectably expressed at P10 (Figure 9A). Based on those preliminary results Tnf, Il1b, and Tgfb1 mRNA were selected for further analysis at E18.5, P5, and P12–P15, while the number of animals analyzed at P10 was increased. We observed moderate 2- to 3-fold up-regulation of Tnf at P5 and P10 in the colon of Gfra1hypo/hypo animals (Figure 9B). Il1b mRNA expression showed increased expression in Gfra1hypo/hypo mice with remarkable interanimal variation (Figure 9C). Expression levels of Tgfb1 were similar between genotypes (Figure 9D). The downward trend in Tnf and Il1b mRNA expression in older animals may reflect the fact that more severely affected individuals die before P10 (Table 1). The observed peak in Tnf and Il1b mRNA expression in a few animals therefore may reflect the end stage of the disease, which is associated with acute bacterial enterocyte attachment, tissue invasion, and sepsis.

In the small intestine we were unable to detect significant differences in Tnf and Il1b mRNA expression (Figure 9E and F).

We also analyzed cytokine interneron γ, interleukin (IL) 12, IL4, IL5, IL6, tumor necrosis factor (TNF), IL1b, and IL9 protein levels in the serum using commercially available enzyme-linked immunosorbent assay (ELISA) analysis tools at P10 and P14–P16. We were able to detect TNF, IL5, and IL6 with no clear genotype–phenotype correlation (Figure 9H and I). We hypothesized that systemic immune activation with notable serum cytokine changes in the Gfra1hypo/hypo HSCR/HAEC mouse model was a late event that was associated with bacterial invasion and sepsis at the end stage.

**Discussion**

Here, we report that reducing GFRα1 expression in mice does not affect renal development, but is sufficient to cause a long-segment HSCR-like phenotype. Why is ENS development in heterozygous Gfra1WT/KO mice grossly normal, whereas homozygous Gfra1hypo/hypo mice, which have only a 20%–30% further reduction in GFRα1 levels compared with Gfra1WT/KO mice, develop HSCR? Our results using a reporter cell line suggest that GFRα1 levels need to exceed a specific threshold before downstream signaling is activated. It is possible that during ENS development this threshold level of GFRα1 in Gfra1hypo/hypo mice is not reached. Previous attempts to alter GDNF/GFRα1/RET signaling by introducing knock-in alleles for Ret led to defects in both the ENS and kidneys.24 It is possible that the level of RET phosphorylation required for normal development is higher in the developing ENS compared with the developing kidneys. GDNF and GFRα1 also can promote synapse formation in a...
Table 3. Summary of Histopathologic Findings at P10

| P10 ID | Weight, g | Sex | Applied Telitbaum et al. grade | Surface epithelial damage | Crypt dilation | Intraepithelial apoptotic remnants | Goblet cell hyperplasia | Mucin shift to PAS | Basal | Surface | Mucus retention in crypts | Bacteria in crypts | Enterocyte adherence | Granulocytes Lymphocytes |
|--------|----------|-----|-------------------------------|--------------------------|--------------|----------------------------------|-----------------------|-------------------|-------|---------|------------------------|-------------------|----------------|-----------------------------|
| WT 10  | 6.8 F    |     | 0                             | -                        | -            | No                               | No                    | AB                | PAS   | -       | -                      | -                 | -              | -                           |
| WT 23  | 5.3 M    |     | 0                             | -                        | -            | No                               | No                    | AB                | PAS   | -       | -                      | -                 | -              | -                           |
| WT 6280-1 | 8 M |     | 0                             | -                        | -            | No                               | No                    | AB/PAS            | PAS   | -       | -                      | -                 | -              | -                           |
| WT 31  | 4.2 M    |     | 0                             | -                        | -            | No                               | No                    | AB/PAS            | PAS   | -       | -                      | -                 | -              | -                           |
| WT/hypo 22 | 6.09 M |     | 0                             | -                        | -            | No                               | No                    | AB/PAS            | PAS   | -       | -                      | -                 | -              | -                           |
| WT/hypo 24 | 6.42 F |     | 0                             | -                        | -            | No                               | No                    | AB/PAS            | PAS   | -       | -                      | -                 | -              | -                           |
| WT/hypo 6777-3 | 5.18 M |     | 0                             | -                        | -            | No                               | No                    | AB/PAS            | PAS   | -       | -                      | -                 | -              | -                           |
| WT/hypo 6280-2 | 7.84 M |     | 0                             | -                        | -            | No                               | No                    | AB/PAS            | PAS   | -       | -                      | -                 | -              | -                           |
| WT/hypo 12 | 6.42 M |     | 0-I                           | -                        | -            | No                               | No                    | AB/PAS            | PAS   | -       | -                      | -                 | -              | -                           |
| Hypo/hypo 11 | 3.61 M |     | I                             | Minimal                | -            | Yes                              | Yes                   | PAS               | PAS   | -       | -                      | -                 | -              | -                           |
| Hypo/hypo 27 | 7.57 M |     | II                            | Mild                   | -            | +                                | Yes                   | Yes               | PAS   | PAS     | -                      | -                 | No             | -                           |
| Hypo/hypo 6277-7 | 3.6 F |     | II                            | Mild                   | +           | +++                              | Yes                   | Yes               | PAS   | PAS     | +                      | No                | -              | -                           |
| Hypo/hypo 21 | 4.93 F |     | III                           | Moderate               | +           | +                                | Yes                   | Yes               | PAS   | PAS     | ++                     | -                 | No             | -                           |
| Hypo/hypo 6224-2 | 4.15 M |     | II                            | Mild                   | +           | +                                | Yes                   | Yes               | PAS   | PAS + AB| +                      | No                | -              | -                           |
| Hypo/hypo 6224-7 | 5.93 F |     | III                           | Moderate               | ++          | +                                | Yes                   | Yes               | PAS   | PAS + AB| +                      | No                | ++             | -                           |
| Hypo/hypo 7310-1 | 4.53 M |     | III                           | Moderate               | ++          | +                                | Yes                   | PAS/foamyAB       | PAS/foamyAB | +++      | -                      | No                | -              | -                           |
| Hypo/hypo 7310-2 | 3.62 M |     | II                            | +++/++++               | -           | Yes                              | Yes                   | PAS/foamyAB       | PAS/foamyAB | +++      | -                      | No                | -              | -                           |

Mineral Mild + Mild + Moderate Minimal Mild + Normal (<4/HPF)

0–V Mild Moderate Moderate Yes/no Yes/no Moderate Yes/no Mild + (5–15/HPF)

00 Moderate Marked Marked Yes/no Yes/no Moderate Yes/no Mild + (20–30/HPF)

NOTE. See the Materials and Methods section for details regarding the histopathologic grading.
RET-independent manner via a process called ligand-induced cell adhesion, which also may influence cell migration, and therefore synapse formation could be another process in which a reduction in GFRa1 levels may impact ENS development differently from the kidneys. Unfortunately, methods for quantifying RET phosphorylation in vivo are not currently available, thus precluding the ability to quantify RET signaling to discriminate between RET-dependent from RET-independent effects in ENS and kidney development.

Our Gfra1hypo/hypo mouse provides an opportunity to model childhood HSCR and HAEC arising from a defect in the GDNF/GFRa1/RET signaling axis. At 10 days of age, Gfra1hypo/hypo mice recapitulate the principal features of childhood long-segment HSCR and the early features of HAEC, including a failure to thrive, long-segment aganglionosis of the colon, the presence of hypertrophic cholinergic fibers in the distal colon, hyperplasia of goblet cells in the colon, mucus retention, a shift from acidic to neutral mucin production, crypt dilatation, and surface epithelial damage. Epithelial damage included focal epithelial necrosis and modest neutrophil infiltration, corresponding to mild cryptitis in human beings. Importantly, although the presence of most of these pathologic changes varied among the Gfra1hypo/hypo mouse colon samples at P4 and P10, 100% of the Gfra1hypo/hypo mice at both ages had a shift from acidic to neutral mucin production. By P10, 100% of Gfra1hypo/hypo mice also showed similar levels of goblet cell hyperplasia, whereas bacterial enterocyte adherence was not observed in any mice at this age. These results suggest that goblet cell dysfunction in the colon precedes the other pathologic changes, and that bacterial enterocyte adherence is not an initiating factor in HAEC. Gfra1hypo/hypo mice also provided us with an opportunity to study whether the observed altered goblet cell physiology results from the lack of the ENS in the colon and distal parts of the small intestine or developmental reduction in GFRa1 in the mesenchyme of the whole fetal intestine. We found that in the duodenum of Gfra1hypo/hypo mice both the goblet cell numbers and the mucin profile are normal, suggesting that colonic goblet cell hyperplasia results from the lack of ENS.

At a later age (P18–P25), all of the Gfra1hypo/hypo mice studied showed previously known features of HAEC, including copious mucus retention, epithelial damage in the colon, goblet cell hyperplasia mixed with goblet cell degeneration, and crypt dilatation and inflammation. Importantly, enterocyte adherence and bacterial invasion were present in only 2 of the 3 mice that had the most advanced epithelial damage, suggesting that enterocyte adherence and bacterial invasion are late events that mark the end stage of the disease. Taken together, our results suggest that HAEC may be associated with goblet cell dysfunction, which manifests first as a shift from acidic toward neutral mucin production in the early stage, followed by hyperplasia and a continuous mucin shift, and mucin overproduction during the later stages. Over time, these features lead to mucus retention and crypt dilatation, which in turn contribute to progressive dysfunction of the intestinal barrier, including progressive epithelial damage and ulceration; at the end stage of the disease, these pathologic changes facilitate bacterial enterocyte adherence, bacterial invasion, and, ultimately, sepsis. Our results shed light on the sequence of events in HAEC and suggest that addressing goblet cell dysplasia, which could remain in the proximal colon or transition zone after surgical removal of the aganglionic gut segment, may be beneficial in treating or preventing HAEC.

Together, mutations in endothelin-related genes that encode EDN3, EDNRB, and ECE1 account for approximately 75% of cases of HSCR and HAEC. However, these mutations do not explain all cases of HSCR and HAEC. This leaves open the possibility that other mutations in genes involved in the ENS or in genes that regulate ENS development may be responsible for the remaining cases of HSCR and HAEC.

Figure 7. Histopathologic analysis of moderate and marked HSCR/HAEC in P18–P25 Gfra1hypo/hypo mice. (A–C) Exacerbated mucin type changes, epithelial damage, scarce inflammation in the lamina propria, and a lack of bacterial invasion in P18–P25 Gfra1hypo/hypo mice with moderate-grade HAEC. (A) In WT mice, the crypts in the colon (A1 and A2, white arrow) are relatively shallow and straight, and the surface epithelium is even and regularly palisading (A1, curved black arrow). The goblet cells in the surface epithelium and in the proximal parts of the crypts contain PAS-positive (ie, neutral) mucins, staining deep purple (A2, white arrowhead). In the basal parts of the crypts, apical mucin droplets in the colonocytes and the mucus in the goblet cells stain primarily with AB, staining light blue (mucin droplets; A2, yellow arrowhead) to dark blue (goblet cells; A2, open arrowhead). In A3, gran-positive (blue to black) and gran-negative (intense red) bacteria are isolated from the surface epithelium by a layer of mucus (A3, black arrow). The intestinal lumens of Gfra1hypo/hypo mouse contain fecal material and copious amounts of mucus with eosinophilic debris of shed epithelial cells (B1–B3, white bold arrows). The crypts were markedly dilated with retained mucin (B1–B3, white arrow). The surface epithelium had diffuse degeneration (B1 and B2, curved black arrow) and an increased number of goblet cells (B1–B3, white arrowhead). Multifocal necrotic and apoptotic colonocytes (B2 and B3, open white arrowhead) and scattered intraepithelial neutrophils (B2, red open arrowhead). (C) The basal lamina propria contains moderately increased numbers of eosinophils (C1, black arrowhead) and mildly increased numbers of lymphocytes (C1, yellow open arrowhead), indicative of an inflammatory process. Note the reduction in AB-stained (ie, acidic) mucin (C2, black open arrowhead) in the cytoplasm of basal crypt colonocytes. Gram staining shows copious amounts of mucus (C3, black bold arrow) and a lack of bacteria in mildly dilated crypts (C3, white arrow). (D and E) Marked epithelial damage at the surface epithelium, crypt abscesses, bacterial invasion, and inflammation in P18–P25 Gfra1hypo/hypo mice with marked HAEC score. (D) In Gfra1hypo/hypo mice, crypt dilatation (D1, white arrow) along with sparse neutrophil infiltrates in basal (D1, red open arrowheads) and apical (D2, red open arrowheads) crypt epithelium. The superficial epithelium shows multifocal necrotic areas and degenerative changes accompanied by superficial dense infiltrates of neutrophils (ie, microabscesses; D2, green arrowheads). Crypt abscesses (E1, green arrow), dense bacterial aggregates (D3, black arrow), and enterocyte-attaching bacteria (D3 and E2, green bold arrow) also are indicated. Bacteria inside a submucosal blood vessel (E3, black arrow) and a bacterial aggregate in mildly dilated crypt base (E3, white arrow) are present. Single granulocytes (E3, yellow bold arrows) and lymphocytes (E3, yellow arrowheads) are present in the lamina propria and submucosa, indicative of inflammation.
5% of all HSCR cases. Various animal models of HSCR with defective endothelin signaling have been generated, and substantial progress has been made using those models toward understanding short-segment HSCR and HAEC. A careful comparison between these endothelin-defective models and our Gfra1hypo/hypo mice is an important future objective. Such a study likely would show similarities—and probably also differences—between the 2 genotypes and might pave the way for the development of gene-specific treatment strategies.

To date, more than 10 HSCR susceptibility genes have been identified. However, together these genes account for approximately 50% of all HSCR cases. Thus, other genetic mutations and/or traits likely underlie HSCR. At present, neither GFRα1 protein nor GFRα1 mRNA levels are routinely measured in colon samples resected from HSCR patients. One study using semiquantitative PCR reported reduced levels of GFRα1 mRNA in 3 of 13 HSCR patients analyzed. Given that our results show that reducing GFRα1 levels is sufficient to cause HSCR in mice, measuring GFRα1 levels in a larger cohort of HSCR patients in conjunction with epigenetic and genomic sequencing analysis is an important future objective.

**Materials and Methods**

**Animals**

Gfra1hypo mice (registered in the Mouse Genome Database as the 129Ola/ICR/C57BL6-Gfra1tm1Joao mouse line) were generated by inserting a puro cassette into intron 6.
### Table 4. Summary of Histopathologic Findings at P18–P25

| P18–P25 ID       | Weight, g     | Sex | Surface epithelial injury | Intraepithelial apoptotic remnants | Goblet cell hyperplasia | Mucins in crypts | Bacteria in crypts | Enterocyte adherence | Leukocytes |
|------------------|---------------|-----|---------------------------|-----------------------------------|-------------------------|----------------|-------------------|---------------------|------------|
|                 |               |     | Crypt dilation            |                                    |                         | Mucin shift to PAS | Basal | Surface | Mucus retention | Enterocyte | Granulocytes | Lymphocytes |
| WT 29.12.8-9    | ND 29.12.8-9  |     | 0                         | -                                 | No                      | No             | AB                | -                  | -          |
| WT 23.3-9-5     | 11.1          | F   | 0                         | -                                 | No                      | No             | AB                | -                  | -          |
| WT 22.9.10CA-2  | 10.1          | F   | 0                         | -                                 | No                      | No             | AB                | -                  | -          |
| WT 5.1.9-9      | 12.23         | F   | 0                         | -                                 | No                      | No             | AB                | -                  | -          |
| WT/hypo 29.12.8-5 | ND 29.12.8-5 |     | 0                         | -                                 | No                      | No             | AB                | -                  | -          |
| WT/hypo 23.3-9-6 | 12.1          | F   | 0                         | -                                 | No                      | No             | AB                | -                  | -          |
| WT/hypo 22.9.10CA-7 | 84            | F   | 0                         | -                                 | No                      | No             | AB                | -                  | -          |
| WT/hypo 22.9.10CA-8 | 8.9           | F   | 0                         | -                                 | No                      | No             | AB                | -                  | -          |
| Hypo/hypo 29.12.8-8 | ND ND         |     | 0                         | -                                 | No                      | No             | AB                | -                  | -          |
| Hypo/hypo 23.9-4 | 9.4           | M   | II                        | Moderate                          | +                       | Yes             | PAS/foamy AB      | +                  | -          |
| Hypo/hypo 22.9.10CA-3 | 8.5          | M   | II                        | Moderate                          | ++                      | Yes             | PAS/foamy AB      | +                  | -          |
| Hypo/hypo 22.9.10CA-10 | 7.8         | F   | II                        | Moderate                          | +++                     | Yes             | PAS/foamy AB      | +                  | -          |
| Hypo/hypo 5.1.9-7 | 8.8           | M   | IV/V                      | Marked                            | ++++                    | Yes             | PAS/foamy AB      | +                  | -          |
| Hypo/hypo 5.1.9-7 | 8.8           | M   | IV/V                      | Marked                            | ++++                    | Yes             | PAS/foamy AB      | +                  | -          |
| Hypo/hypo 5.1.9-7 | 8.8           | M   | IV/V                      | Marked                            | ++++                    | Yes             | PAS/foamy AB      | +                  | -          |

NOTE. See the Materials and Methods section for details regarding histopathologic grading. ND, not determined.
of the Gfra1 gene using gene targeting via homologous recombination in embryonic stem cells (Figure 1A–C). Embryonic stem cell clones that had undergone homologous recombination in 1 Gfra1 allele were identified by performing Southern blot analysis on EcoRV-digested genomic DNA using the probe indicated in Figure 1A (expected sizes: targeted, 7564 bp; WT, 10397 bp). Routine PCR-based genotyping of the offspring was performed using the primers indicated in Figure 1A (GfraloxF forward: 5′-cattgccaggtggaaagaca; Gfra1loxR reverse: 5′-agaaagaga gatgacagctacattg). The mice were maintained on a 129Ola/ICR/C57BL6 mixed genetic background, housed under a 12-hour/12-hour light/dark cycle at 20°C–22°C, with 1 mother and litter per cage; standard chow and water were available ad libitum. All animal experiments were approved by the National Animal Experiment Board of Finland.

**Tissue Processing**

Paraffin-embedded sections, whole-mount GI tracts (E13.5), and myenteric plexuses (P10 or P18–P25) were used for histologic preparations. Comparable samples obtained from the duodenum, ileum, and colon were analyzed from all genotypes, using littermates as controls. The samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 hours at room temperature. Automated dehydration of the tissues and subsequent paraffin embedding were performed using an ASP300 S tissue processor (Leica, Wetzlar, Germany). Paraffin blocks were cut to prepare 5-μm sections using a Tissue-Tek microtome (Sakura, Alphen aan den Rijn, The Netherlands). For whole-mount histology of P10 and P18–P25 tissues, the mice were perfused intracardially with PBS and 4% paraformaldehyde. The longitudinal muscle/myenteric plexus (LMMP) preparations were isolated under a dissection microscope by peeling off the outer muscle layer of the GI tract and postfixed for 15 minutes in paraformaldehyde.

**Histopathology**

Paraffin sections of the duodenum and colon were stained with H&E, Gram stain, or a combination of AB (pH 2.5) and PAS using standard protocols. LMMPs from the small and large intestine were stained for acetylcholines terase as previously described. Briefly, LMMP was washed in PBS for 1 hour, 100 mmol/L maleic acid buffer pH 6.0 for 5 minutes, pre-incubated for 30 minutes in 65 mmol/L maleic acid buffer including 0.5 mol/L potassium ferricyanide, 4 mmol/L copper sulfate, and 5 mmol/L sodium citrate, and reacted for 30–45 minutes in 5 mmol/L ace tylthiocholine iodide in 100 mmol/L maleic acid buffer. NADPH diaphorase was used to visualize nitrergic neurons in LMMPs; briefly, LMMPs were washed 2 times 1 hour in PBS and reacted 1 hour at +37°C in a solution prepared right before use containing 0.3% Triton (Sigma) X-100, 0.01% nitro blue tetrazolium chloride, and 0.1% NADPH in PBS. Finally, the LMMP preparations were washed 6 times for 5 minutes in PBS and mounted in glycerol for imaging.

**Histopathology Grading**

The criteria for histopathologic grading are defined later. HAECA-associated inflammatory cell infiltration of the crypts (cryptitis and crypt abscesses) was milder in mice than in human beings, whereas epithelial pathology characteristic of HAECA was recapitulated well in mice. Therefore, we modified the grading system reported by Teitelbaum et al7 to primarily reflect epithelial pathology.

*Surface epithelial damage.* Epithelial damage was graded as minimal, mild, moderate, or marked.

**Minimal surface epithelial damage.** The surface epithelium consists of mild disorganization and slight degeneration of the surface epithelium. In contrast to regular palisading, colonocytes are clustered multifocally in disorganized, undulating rows with partially overlapping nuclei. Apical eosinophilia and indistinct cell borders are also visible focally.

**Mild surface epithelial damage.** The surface epithelium has mild to moderate disorganization and mild degenerative changes. The size and shape of the epithelial cells and nuclei vary, and they grow in disorganized, undulating rows with partially overlapping nuclei. Apical cytoplasms are generally homogenously eosinophilic or vacuolar, and the cell borders are indistinct; cells are occasionally low columnar to cuboidal in shape. Scattered intraepithelial apoptotic/necrotic cell remnants are present.

**Moderate surface epithelial damage.** The surface epithelium has mild to moderate disorganization and intense staining. Increased numbers of apoptotic/necrotic cells or cell remnants are present, as well as clusters of apoptotic/necrotic cells. Scattered intraepithelial neutrophils (1–5 per high-power field [HPF]) may be present in the lamina propria. Mildly increased numbers of lymphocytes in the basal lamina propria (indicative of mild chronic colitis) were detected in 1 mouse; however, in accordance with a previous report on HAECA in mice, inflammatory activity is generally very low.

**Marked surface epithelial damage.** The surface epithelium has generally diffuse degeneration, and multiple

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**Figure 9. (See previous page). Analysis of cytokine mRNA and protein levels.** (A–D) qPCR analysis of cytokine mRNA levels in the colon of Gfra1^hypo/hypo^ and control mice at indicated ages. Tnf mRNA levels are up-regulated in P5 colon (P < .05, analysis of variance and Tukey multiple comparisons) and in P10 colon (P < .05, analysis of variance and Tukey multiple comparisons). (E and F) Tnf and Il1b mRNA levels are not up-regulated in the small intestine (a segment spanning the duodenum–jejunum was analyzed). (G–I) Measurements of serum cytokine protein levels by ELISA. In analysis of Gfra1^hypo/hypo^ and control mice at the indicated ages, no significant differences between the genotypes were observed. The number of animals is given for each time point in the Figure panels (qPCR: 2–3 biological replicates, 1–2 independent experiments per animal; ELISA: 2 biological replicates, 1 experiment per animal). All summary data are presented as means ± SEM. *P < .05, **P < .01, and ***P < .001.
necrotic areas accompanied by small superficial infiltrates of neutrophils (microabscesses) are present. The apical parts of the crypts of Lieberkühn contain few to several intraepithelial neutrophils with sporadic crypt abscesses. The goblet cells are degenerated. The lamina propria contains mildly to moderately increased numbers of eosinophils and neutrophils in the basal areas and between the crypts. The inflammatory reaction in the intestinal wall is mild.

**Loss of integrity of the gut wall/bacterial invasion.** The surface epithelium shows gross changes of varying degrees. The lamina propria has mild to moderate edema and mildly to moderately increased numbers of eosinophils, neutrophils, and lymphocytes. Profuse bacterial accumulates are present in some of the moderately to markedly dilated crypts of Lieberkühn. The submucosa has marked edema, sparse infiltrates of leukocytes, and small numbers of bacteria. The circular and longitudinal muscle layers have marked diffuse degeneration and moderate neutrophilic infiltrates. Mesothelial cells are reactive.

**Crypt dilatation.** Crypt dilatation was graded as minimal to mild (+), moderate (++), or marked (+++) to quantify the extent of the changes. In cases in which large segmental variation was present, both grades were reported.

**Mild crypt dilatation.** The dilatation is present primarily at the base of the crypts, and any from a few to approximately half of the crypts are affected.

**Moderate crypt dilatation.** Most of the crypts are affected.

**Marked crypt dilatation.** The apical areas of the crypts also are affected, and most of the crypts are markedly dilated.

**Leukocyte numbers in the mucosa.** A 3-tier system was used to grade leukocyte numbers as minimal to mild (+), moderate (++), and marked (+++).

**Lymphocytes.** Lymphocyte number is considered to be normal if 5 or fewer cells are generally present in the lamina propria between the crypts per HPF. A minimal to mild increase in lymphocytes is scored if they fill the intercryptal region, and they may be increased basally below the crypt bases. There is no increase in separation of the crypts. A moderate increase is scored if the lymphocytes increase the separation of the crypts; a marked increase is scored if there is a diffuse distribution of lymphocytes with distortion of the cryptal architecture. In accordance with a previous report of HAEC in mice, none of our samples contained moderate or marked increases in lymphocytes. Accordingly, extremely few plasma cells were detected, and plasma cells therefore were excluded from grading.

**Granulocytes.** Eosinophils and neutrophils were graded as a single entity. Neutrophils related to surface epithelial damage (eg, crypt abscesses, microabscesses in the epithelium, and epithelial neutrophils) were omitted from this section and were graded under surface epithelial damage.

Granulocyte number is considered to be normal if no more than 3–4 granulocytes typically are present per HPF in the lamina propria. A minimal to mild increase is scored if approximately 15 granulocytes are present per HPF in the lamina propria. A moderate increase is scored if the infiltrate contains 20–30 granulocytes per HPF; this may be accompanied by macrophages. A marked increase is scored if granulocytes are the dominant population and are not easily counted per HPF.

**Quantification of Goblet Cells**

Goblet cells were counted in the distal third of the colon from the basal half of the crypts. Twenty to 30 crypts were counted per sample. Goblet cells that primarily were stained light blue were counted as AB+ and goblet cells stained primarily magenta or purple were counted as PAS+. Goblet cells fitting neither category were counted as AB-PAS+. Sample genotypes were blinded for the counter.

**Immunohistochemistry**

Sections were deparaffinized through serial washes with xylene, alcohol, and water. Antigen retrieval was performed by boiling the samples for 10 minutes in citrate buffer (10 mmol/L sodium citrate, 0.05% Tween 20, pH 6.0). Endogenous peroxidase activity was quenched in 1:53 H2O2 standard solution in Tris-buffered saline (TBS) for 30 minutes at room temperature. After washing with TBS containing 0.1% Tween 20, the tissue was blocked in 1.5% normal goat serum in TBS containing 0.1% Tween 20 for 30 minutes at room temperature. The sections were incubated overnight at 4°C in primary antibody solution containing rabbit anti-ubiquitin C-terminal hydrolase L1 (1:250, BML-PG9500; Enzo Life Sciences) or rabbit anti-glial fibrillary acidic protein (1:200, Ab-4 RB-087-A; ThermoScientific). After washing, the sections were incubated for 90 minutes at room temperature in secondary antibody solution containing biotinylated anti-rabbit antibody (1:200; Vector Laboratories) or goat anti-rabbit Alexa 488 antibody (1:400, A11034; Invitrogen). The biotinylated secondary antibody signal was enhanced using the ABC reaction kit (PK-4001, Vector Laboratories) and visualized using 3,3′-diaminobenzidine (SK-4100, Vector Laboratories). Samples were dehydrated through serial washes with water, alcohol, and xylene, and mounted using Depex (Science Services GmbH, München, Germany). Fluorescence-labeled, whole-mount samples were mounted in glycerol and imaged using an Olympus BX-61 microscope. As negative controls, either the primary or secondary antibody was omitted.

**ELISA**

ELISA was performed with commercial kits according to the manufacturer’s instructions (Invitrogen ProcartaPlex multiplex interferon γ, IL12, IL4, IL5, IL6, TNF, EPX060-20831-90, IL1B Mouse ProcartaPlex Simplex Kit, EPX01A-26002-901, and IL9 Mouse ProcartaPlex Simplex Kit, EPX01A-26041-901).

**Quantitative PCR**

**RNA isolation.** RNA was isolated from snap-frozen tissues using the TRIzol reagent (Invitrogen) for samples collected from E18.5 on or the RNAqueous Micro-kit (AM1931; Life Technologies) in accordance with the manufacturer’s instructions. DNase I was supplied in the same kit.

**Reverse transcription.** First-strand complementary DNA (cDNA) was synthesized from 50 to 300 ng of RNA (with
equal amounts of starting RNA used in each experiment) using random hexamer primers in a final volume of 20 μL (Transcriptor First-Strand cDNA synthesis kit; Roche). In brief, 2 μL of random hexamer primers was mixed with 11 μL of RNA sample diluted in nuclease-free water and incubated at 65°C for 10 minutes. Next, 7 μL of a mixture containing 4 μL of 5× reverse transcriptase buffer, 2 μL of 100 mmol/L deoxynucleoside triphosphate, 0.5 μL of RNase inhibitor, and 0.5 μL of Transcriptor reverse transcriptase (Transcriptor First-Strand cDNA synthesis kit; Roche) were added, mixed gently, and incubated at 25°C for 10 minutes, 55°C for 30 minutes, and 85°C for 5 minutes. A control sample containing no reverse transcriptase was included in each experiment. The cDNA was cooled on ice, diluted 1:15 in water, and either stored at -20°C or used immediately for quantitative PCR (qPCR).

**Real-time qPCR.** qPCR was performed using the LightCycler 480 real-time PCR system (Roche Diagnostics) with the LightCycler 480 SYBR Green I Master mix and 2.5 pmol of primers at a final volume of 20 μL. The reactions were run on white 384-well plates sealed with adhesive plate sealers (04729749001; Roche Diagnostics). Each reaction contained 2.5 μL of diluted cDNA. Each qPCR run contained 2–3 replicates of each reaction. The following qPCR program was used: pre-incubation: 10 minutes at 95°C; amplification: 10 seconds at 95°C, 15 seconds at 60°C, and 15 seconds at 72°C for 45 cycles; melting curve: 5 seconds at 95°C, 30 seconds at 55°C, continuous acquisition mode at 95°C with 2 acquisitions per degree Celsius; and cooling: 10 seconds at 40°C. The data were analyzed using LightCycler 480 Software Release 1.5.0 SP1, with the Absolute Quantification/2nd Derivative Max calculation. Actb was amplified as a reference gene. The following *mus musculus* (m) primer sets were used in this study: mActb: 5’-actagggcaccagctgagaaag and 5’-accagagcatacaagggaca; mGdnf: ex2–3: 5’-cgctgctgagctcctcatggca; mGfra1: 5’-gttc cacacagtttacca and 5’-gcccacctattgattcctttgcttgcttgcttgcttgcttggtcag; mIl1b: 5’-agttgacagcaccacaaag and 5’-agttgacagcaccacaaag; mIl1a: 5’-gtcgc ccacaaaagattg and 5’-gtgcctcattgttacctgaag; mIl1Ra: 5’-aa caccctgtcctgccctgactta and 5’-gcccaagaacacctagtaagagct; mIl2: 5’-ttgggttctttttgacacagcttgacagtaag and 5’-ttgggttctttttgacacagtaagagct; mIl4: 5’-aa cgagtttcacaggagaaag and 5’-tctgacatcagcagaaag; mIl5: 5’-acaggttacatcagtaacag and 5’-tctcgtccaatcttttctttt; ml6: 5’-accacttcaccaagttgcgag and 5’-tgcaagtctgcaatggttg; ml110: 5’-ataactg gcaacatccttccca and 5’-ttggccacaagtaacctccacacccc; ml13: 5’-gcgc caccagttggtgtgtgg and 5’-tgccaagacagctctctgctgcttgcttgcttgcttgcttggtcag; mRet et2: 5’-tccc ttccacagtcttgaag and 5’-atggctcctctctctctcctcctgatg; mTgfβ1: 5’-tgagc gcacatgttgatc and 5’-tgtacacgctcatgccacatc; and mTnf: 5’-gatc ggtcctccaaaggctgag and 5’-tggagtgtcgttggcctcatg.

**Western Blot Analysis**

Snap-frozen E12.5 tissue was homogenized in a homogenization solution containing 0.3 mol/L sucrose, 10 mmol/L HEPES, 1 mmol/L EDTA (pH 7.2–7.4), and a proteinase inhibitor (Complete Mini-Tabs Cocktail Set; Roche); the protein concentration was measured using the Lowry method (Bio-Rad). A total of 5–10 μg of protein was mixed with Laemmli sample buffer and incubated at 95°C for 5 minutes. The proteins were separated on an 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Protran, GE Healthcare). After washing and blocking at room temperature for 60 minutes in 5% milk, the membranes were incubated in goat anti-Gfra1 antibody (1:1000, GT15004; Neuronics). After washing, the membranes were incubated at room temperature for 2 hours in horseradish peroxidase–conjugated anti-goat antibody (1:2000, P0449; Dako). The protein bands were visualized using enhanced chemiluminescence substrate (Pierce). As a loading control, the membranes then were stripped and reprobed using anti-α-tubulin antibody (1:60,000, T9026; Sigma), followed by horseradish peroxidase–conjugated donkey anti-mouse antibody (1:2000, P0449; Dako). Three independent Western blots were quantified with ImageJ 1.50a software (National Institutes of Health, Bethesda, MD) and an unpaired Student t test was performed as a statistical analysis using GraphPad Prism software.

**Luciferase Assay**

RET-dependent activation of mitogen-activated protein kinase by soluble GFRα1 was measured using MG68 fibroblasts that stably express RET (MG87RET) and the PathDetect-Elk1 system (219005; Stratagene). One day before the assay, cells were plated in a 96-well plate (20,000 cells/well) in 100 μL/well Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and 100 ng/mL normocin (ant-nr-1; InvivoGEN) and cultured overnight in an incubator with 5% CO₂. Soluble GFRα1 (1–5000 ng/mL, 560–GR; R&D Systems) and GDNF (100 ng/mL 450–10; Peprotech) were added to wells in quadruplicate per GFRα1 concentration. The plates then were cultured for an additional 22–24 hours for luciferase expression, after which the culture media was discarded and the cells were incubated with Neolite reagent (6016711; Perkin Elmer) for 10 minutes. Luciferase activity was measured using a MicroBeta 2 (PerkinElmer) plate counter. Each experiment was performed twice, and the results were analyzed using GraphPad Prism software.

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