Gfra1 under-expression causes Hirschsprung's disease and associated enterocolitis in mice

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**Healthy gut**

- Epithelial cell
- AB+ goblet cell
- PAS+ goblet cell
- Dying cell
- Immune cell
- Neuron
- Bacterium
- Blood vessel
- Mucus

**Mid stage HSCR/HAEC**

- Mucin retention
- Crypt dilation
- Shift in the mucin profile
- Epithelial damage
  \[\rightarrow\] Goblet cell hyperplasia

**Late stage HSCR/HAEC**

- Inflammation
- Loss of epithelial integrity
- Bacterial invasion
- Sepsis
Gfra1 under-expression causes Hirschsprung’s disease and associated enterocolitis in mice

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Reduced Gfra1 causes HSCR and HAEC in mice
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Short title

Gfra1 under-expression causes HSCR and HAEC in mice

Synopsis

70-80% under-expression of Gfra1 results in a Hirschsprung’s disease and associated enterocolitis phenotype in a new mouse model. Enterocolitis proceeds from goblet cell dysplasia, with mucin abnormalities, to epithelial damage. Reduced expression of GFRα1 may contribute to the HSCR susceptibility.

AUTHOR CONTRIBUTIONS

L. L. Porokuokka: acquisition, analysis and interpretation of data; drafting of the manuscript, statistical analysis

H. T. Virtanen: acquisition, analysis and interpretation of data

J. Linden: acquisition, analysis and interpretation of histopathological data; drafting of the manuscript

Y. A. Sidorova acquisition, analysis and interpretation of data; drafting of the manuscript

T. Danilova acquisition, analysis and interpretation of data

M. Lindahl: analysis and interpretation of data, study design, critically read the manuscript

M. Saarma: initiated the in vivo GFRα1 studies; critically read the manuscript; provided funding

J-O. Andressoo: designed and generated Gfra1hypo mice; acquisition and interpretation of data; study design; drafting of the manuscript; provided funding

MS and JOA contributed equally to this work

The authors declare no conflicts of interest.

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Abbreviations:

1. **AB-PAS** Alcian Blue - Periodic Acid Shiff
2. **AChe** Acetyl choline esterase
3. **ECE1** Endothelin converting enzyme 1
4. **EDNRB** Endothelin receptor type B
5. **EDN3** Endothelin 3
6. **ENCC** Enteric neural crest-derived cell
7. **ENS** Enteric nervous system
8. **GDNF** Glial cell line-derived neurotrophic factor
9. **GFAP** Glial fibrillary acidic protein
10. **GFRa1** GDNF family receptor a1
11. **GI** Gastrointestinal
12. **GPI** Glycosylphosphatidylinositol
13. **HAEC** Hirschsprung’s disease associated enterocolitis
14. **HSCR** Hirschsprung’s disease
15. **IFN** Interferon
16. **IL** Interleukin
17. **KIF1BP** KIF1 binding protein
18. **LMMP** longitudinal muscle/ myenteric plexus preparation
19. **MAPK** Mitogen-activated protein kinase
20. **NOS** Nitric oxide synthase
21. **NRTN** Neurturin
22. **PHOX2B** Paired-like homeobox 2b

Reduced Gfra1 causes HSCR and HAEC in mice
Abstract

Background & Aims: RET, the receptor for the 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 HSRC-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 postnatally viable mouse model for HSCR/HAEC and analyze HAEC etiology.

Methods: GFRα1 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.

Reduced Gfra1 causes HSCR and HAEC in mice
Results: We report that 70-80% reduction in GDNF co-receptor GFRα1 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 histopathological findings in children with untreated HSCR/HAEC.

Conclusions: In GFRα1 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 are 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 GFRα1 may contribute to the HSCR susceptibility.

Keywords: aganglionosis; megacolon; transgenic mouse model
Introduction

Hirschsprung’s disease (HSCR, also known as congenital intestinal aganglionosis) is a congenital malformation characterized by a lack of enteric ganglia in the distal part of the gut leading to constipation, gut distention, and associated failure to thrive. The underlying pathogenic etiology is widely accepted to be based on defects in the craniocaudal migration, proliferation, differentiation, and survival of neuroblasts that originate from the neural crest in the early stage of pregnancy [1, 2]. The prevalence of HSCR is 1 in 5000 live births, and the disease is generally subdivided into short-segment and long-segment types based on the point at which histologically characterized aganglionosis begins. Currently, HSCR is routinely 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 [3]. HAEC develops in approximately 30% of patients with short-segment and 50% of patients with long-segment HSCR [4, 5]. The incidence of HAEC suggests either multigenic and/or environmental contribution [4, 6], but the pathogenesis of HAEC remains poorly understood. Mucosal barrier dysfunction as a predisposing factor is one of the several proposed drivers for HAEC [4-6]. HAEC is clinically characterized by fever, abdominal distention, diarrhea, and sepsis. Histopathological features in the colon include crypt dilatation, mucin retention, enterocyte adherence of bacteria, a shift from acidic towards neutral mucin production, epithelial damage, leukocyte infiltration, ulceration, and, in the terminal stages, transmural necrosis and perforation [4-7]. However, at least in part due 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.

Reduced Gfra1 causes HSCR and HAEC in mice
The most common genetic cause of HSCR is the presence of inactivating mutations in the receptor tyrosine kinase RET, the signaling receptor for glial cell line-derived neurotrophic factor (GDNF); approximately 50% of cases are familial, and 15-35% are sporadic cases [2, 8-11]. In addition, at least 13 other HSCR susceptibility genes have been identified, including GDNF, neurturin (NRTN), endothelin receptor type B (EDNRB), endothelin 3 (EDN3), endothelin-converting enzyme 1 (ECE1), Sry-like HMG box 10 family member (SOX10), paired-like homeobox 2b (PHOX2B), KIF1-binding protein (KIF1BP), zinc finger E-box binding homeobox 2 (ZEB2), transcription factor 4 (TCF4), and transcription termination factor 1 (TTF1); together, mutations in these genes account for approximately 20% of all HSCR cases [2, 9, 12, 13]. HSCR penetrance is also known to be influenced by gene expression levels since copy number variants of MAPK10, ZFHX1B, and SOX2 loci associate with HSCR in a pilot study on 67 candidate genes [12]. Currently, the underlying genetic cause is unknown for approximately half of all sporadic cases of HSCR [2, 9].

RET is activated upon binding with a complex comprised of GDNF and GDNF family receptor alpha 1 (GFRα1), a glycosylphosphatidylinositol-anchored protein [14]. 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 rostro-caudally along the gut towards a GDNF source, reach the proximal colon at E12, and colon colonization is complete by E14.5 [15, 16]. In addition to regulating ENCC migration, GDNF signaling also controls the proliferation, differentiation, and survival of ENCCs [17-19]. Mice that lack expression of genes encoding GDNF, GFRα1, or RET have a common phenotype Reduced Gfra1 causes HSCR and HAEC in mice
that includes kidney agenesis and a lack of enteric ganglia distal to the stomach [20-22]. 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 [23]. Mouse studies using knock-in or timed conditional deletion alleles for Gfra1 or Ret support the importance of GFRa1/RET signaling in ENS development and survival through postnatal day 1 (P1) [18, 24, 25]. However, animal models with defective GDNF/GFRa1/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 GFRα1 have not been found. Here, we report the generation of GFRα1 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 GFRα1 hypomorphic (Gfra1\textsuperscript{hypo/hypo}) mice have a 70-80% reduction in Gfra1 expression in the developing gut and kidney. Though the kidneys develop normally, Gfra1\textsuperscript{hypo/hypo} 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\textsuperscript{hypo/hypo} mice does lead to HSCR. In addition, our analysis of Gfra1\textsuperscript{hypo/hypo} 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 Reduced Gfra1 causes HSCR and HAEC in mice
an early, ubiquitous event that precedes the epithelial changes, including crypt dilatation, mucin retention, and epithelial damage. Together, these findings suggest that goblet cells could be a candidate target for developing preventative therapies for HAEC and that reduced expression of GFRα1 may represent a novel susceptibility trait for HSCR.

Reduced Gfra1 causes HSCR and HAEC in mice
Results

Generation and primary characterization of \textit{Gfra1}^{hypo/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 [26-30]. We inserted a pu\Delta tk cassette [31] into intron 6 of the \textit{Gfra1} gene (Figure 1A-C) and analyzed the phenotype of homozygous (\textit{Gfra1}^{pu\Delta tk/pu\Delta tk}) mice generated by crossing two heterozygous animals. Compared to their expected Mendelian inheritance, homozygous mice were underrepresented by approximately 3-4 fold at the time of weaning (P18-P25); moreover, homozygous mice developed severe abdominal swelling, failed to thrive, displayed increasing inactivity, and exhibited other signs of discomfort. Of the eight homozygous mice that were monitored beginning at P15, none survived beyond P27; due to ethical considerations, we did not proceed with subsequent survival studies. During further experiments, animals that exhibited 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 revealed marked caudal gut distention (Figure 1D) starting between the distal ileum and the mid-colon. Four out of eight 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 Gfra1, Ret, and Gdnf expression

Quantitative real-time PCR analysis performed at E13.5 revealed that Gfra1 mRNA levels were reduced by 75-80% in the kidneys, stomach, and duodenum of Gfra1<sup>hypo/hypo</sup> mice; expression levels decreased further towards the colon, where Gfra1 mRNA levels were reduced by approximately 90% compared to control animals (Figure 1E). Ret mRNA levels were normal in the stomach and duodenum, but sharply decreased in the ileum of Gfra1<sup>hypo/hypo</sup> mice. In the caecum and colon, Ret mRNA was not detectable in our analysis (Figure 1E). This is expected, since in the developing gut, Ret is expressed exclusively in ENCCs, whereas Gfra1 is expressed both in ENCCs and in the surrounding mesenchyme [16, 32-34]. As our parallel analysis revealed, ENCCs in Gfra1<sup>hypo/hypo</sup> mice are missing distal from mid-ileum (see below).

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 revealed that the amount of GFRα1 protein in the gut segment from the duodenum to the ileum of Gfra1<sup>hypo/hypo</sup> mice was reduced by 75-80% at E13.5 (Figure 1F-G). In the caecum and colon, GFRα1 protein was not detectable in our analysis. Kidney development in Gfra1<sup>hypo/hypo</sup> mice was generally normal (Figure 1H-I). Given the hypomorphic Gfra1 mRNA and protein levels measured in these mice, we renamed the Gfra1<sup>pu/ltk</sup> allele the Gfra1 hypomorphic (Gfra1<sup>hypo</sup>) allele.

Homozygous Gfra1<sup>hypo/hypo</sup> mice have impaired neuronal progenitor colonization and develop aganglionosis and hypertrophic fibers in the colon

Mice that lack Gdnf, Gfra1, or Ret expression lack enteric nerves distal to the stomach [20-22]. Moreover, experiments in which Gfra1 or Ret expression was deleted under temporal control in
the developing gut revealed 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 [18, 25]. Consistent with these findings, innervation of the stomach in Gfra1<sup>hypo/hypo</sup> mice appeared normal at E13.5, and the number of neuronal progenitors decreased gradually towards the distal gut (Figure 2A). At E13.5, Gfra1<sup>hypo/hypo</sup> embryos lacked neuronal progenitors from the mid-ileum onwards (Figure 2A). In the developing gut, Ret is expressed exclusively in ENCCs, whereas Gfra1 is expressed both in ENCCs and in the surrounding mesenchyme [16, 32-34]. As shown in Figure 2A, neuronal progenitors in the distal gut in Gfra1<sup>hypo/hypo</sup> embryos are missing, explaining the observed craniocaudal reduction in Gfra1 mRNA and the absence of Ret mRNA in the distal gut (Figure 1E). Similar innervation defects were observed at E15.5 (i.e., 3-4 days before birth in mice) (Figure 2F).

Heterozygous Gfra1 null-allele (Gfra1<sup>WT/KO</sup>) mice, which have about a 50% reduction in GFRα1 expression, have reduced enteric neuron fiber density and defects in gut function measured ex vivo; however, they do not have reduced numbers of enteric neurons, nor do they develop symptoms of either HSCR or HAEC [20, 23, 35]. Our finding that a 70-80% reduction in Gfra1 expression causes long-segment HSCR suggests a threshold effect in GFRα1-mediated downstream signaling. In the developing gut, two forms of the GFRα1 protein are present—the membrane-associated GPI-anchored form and the extracellular soluble form; these two forms can function cooperatively in mediating GDNF signaling in ENCCs [34]. 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 MG87
fibroblast model system that stably expresses both Ret and a luciferase gene that reflects the activity of mitogen-activated protein kinases (MAPK) [36], 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 nM (Figure 2B). These results provide a possible explanation for the phenotype differences observed between $Gfra1^{WT/KO}$ and $Gfra1^{hypo/hypo}$ mice—in $Gfra1^{WT/KO}$ mice, the GFRα1 levels are within the effective range, whereas GFRα1 levels fall below the effective range in $Gfra1^{hypo/hypo}$ mice.

Long-segment HSCR is defined as aganglionosis proximal to the splenic flexure [37]. Another hallmark clinical feature of HSCR is the presence of extrinsic hypertrophic nerve fibers in the distal colon [38-40]. At E18.5, $Gfra1^{hypo/hypo}$ mice had varying-length aganglionic segments proceeding from the mid-colon (long segment HSCR) or even from the distal ileum (total colonic aganglionosis), and they had hypertrophic nerve fibers in the distal colon (Figure 2C-D). Because the presence of hypertrophic fibers in the colon of HSCR patients is diagnosed postnatally, we analyzed the colon in postnatal (P20) $Gfra1^{hypo/hypo}$ mice and found that the hypertrophic fibers were still present (Figure 2E).

$Gfra1^{hypo/hypo}$ 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 below), 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). Immunohistological analysis of the ENS in $Gfra1^{hypo/hypo}$ mice at P10 using the pan-
neuronal marker PGP9.5 revealed normal ganglia in the duodenum, reduced ganglia size in the
distal ileum, and absent or rudimentary PGP9.5-positive structures in the colon, reflecting
hypertrophic fibers in the colon (Figure 3A-B). Similar results were obtained using the glial
marker GFAP (Figure 3C-D). Collectively, results presented on Figure 2-3 are consistent with
previous results on timed deletion of GFRα1 and RET until E18.5 [18, 25] and suggest that
reduced Gfra1 expression in Gfra1hypo/hypo mice causes impaired craniocaudal 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 nitrergic
stimuli [42, 43]. To examine the properties of the myenteric plexus in Gfra1hypo/hypo mice, we
stained for acetylcholinesterase. Our analysis revealed 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 mid-colon, and hypertrophic cholinergic
fibers in the distal colon (Figure 4A-B). Next, we stained the myenteric plexus for NADPH
diaphorase, a marker of nitric oxide synthase (NOS)–positive nitrergic enteric neurons. Our
analysis revealed 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-D), reminiscent of the histopathological findings in patients with HSCR [42,
43].
Histopathological characterization of HSCR/HAEC in Gfra1<sup>hypo/hypo</sup> mice

Improving our understanding of the histopathological changes that occur in HAEC may eventually help in developing more effective treatments. We utilized the histopathological grading system for HAEC developed by Teitelbaum et al. [7] to analyze Gfra1<sup>hypo/hypo</sup> mice. Teitelbaum et al. scored the histopathological findings in HAEC in patients as follows: 0, no abnormalities; I, crypt dilatation and mucin retention; II, cryptitis or two crypt abscesses per high-power field; III, more than two crypt abscesses per high-power field; IV, fibrinopurulent debris and mucosal ulceration; V, transluminal necrosis or perforation [7]. However, compared to patients, mice with HAEC have milder infiltration of inflammatory cells into the crypts (milder cryptitis and crypt abscesses) [44], although the epithelial pathologies are similar (see below); therefore, we modified the grading system to primarily reflect epithelial pathology (see Methods). 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 hyperplasia [46] and altered goblet cell function [47] have been reported in HSCR.

Histopathological characterization of HSCR/HAEC in E18.5, P4, and P10 Gfra1<sup>hypo/hypo</sup> mice

Histological analysis of the colon in E18.5 Gfra1<sup>hypo/hypo</sup> mice revealed no overt changes compared to control mice (Figure 5A, B). However, at P4, histological analysis using Alcian blue (AB), periodic acid–Schiff (PAS), H&E, and Gram staining revealed from 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 are more advanced: slight to mild multifocal to diffuse epithelial hyperplasia, as well as dysplasia (i.e., disorganization) accompanied by degeneration of surface colonocytes in Gfra1<sup>hypo/hypo</sup> mice (Figure 6B) 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-C). In addition, the crypts in the colon were mildly to moderately dilated with various degrees of mucin retention (Figure 6B-C), indicating an overproduction and retention of mucus in the colon. We also observed increased numbers of intraepithelial apoptotic cell remnants in five out of eight Gfra1<sup>hypo/hypo</sup> mice, indicating a degenerative process (Figure 6B). Importantly, the severity of these features varied between animals (the histopathological data from each animal is 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 above 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 Gfra1<sup>hypo/hypo</sup> colon (Figure 6B, C). On the other hand, AB, which stains acidic mucins, revealed 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 Gfra1<sup>hypo/hypo</sup> colon (Figure 6B-C; Table 3). To gain insight into whether altered goblet cell physiology in the Gfra1<sup>hypo/hypo</sup> 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-H).

Finally, both Gram and H&E staining revealed no evidence of bacterial enterocyte adherence or
microbial infection in the Gfra1<sup>hypo/hypo</sup> colon at P10 (Table 3). Based on our histopathological
 grading system of HAEC, three of the eight Gfra1<sup>hypo/hypo</sup> mice at P10 had a grade III HAEC
score, four mice had grade II HAEC score, and one mouse had a grade I HAEC score.

Taken together, these results suggest that mucin shift from acidic to neutral in goblet cells at P4
is further enhanced and accompanied with hyperplasia at P10 and that those are generally
ubiquitous early events in Gfra1<sup>hypo/hypo</sup> mice. On the other hand, the inter-animal 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.

Histopathological characterization of HSCR/HAEC in P18-P25 Gfra1<sup>hypo/hypo</sup> mice

To analyze the features of advanced HAEC in our Gfra1<sup>hypo/hypo</sup> mice, we examined P18-P25
mice that displayed obvious signs of discomfort, including cachexia, abdominal swelling, and
reduced spontaneous activity. In these mice, we observed histopathological features that are
reminiscent of advanced HAEC in HSCR patients [5-7] (Figures 7 and 8; Table 4). Compared to
P10 mice, in all animals analyzed 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, 7, and 8; Tables 3 and 4). The
shift from acidic to neutral mucin production observed at P10 was also clearly evident at P18-P25 (Figure 7). Moreover, goblet cell hyperplasia (Figure 7B-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 three of the five animals investigated (Figure 7D-E). Bacterial enterocyte adherence on the other hand was only observed in two of the three mice with severe epithelial damage (Table 4); these same two mice also had bacteria within the colon crypts and blood vessels (Figures 7D-E and 8B-C), indicating sepsis. The histopathological 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 Gfra1<sup>hypo/hypo</sup> animals recapitulate both the early and advanced histopathological findings present in patients with HAEC [4, 5, 7].

**Characterization of cytokine mRNA expression and serum protein levels in Gfra1<sup>hypo/hypo</sup> mice**

A mouse long segment HSCR/HAEC model may allow identification of diagnostic markers and further explore relationship between immune response and HAEC. As a first step in this direction we analyzed expression of mRNAs for Il1α, Il1β, Il1Ra, 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. Out of 13 selected mRNAs seven 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 P12-15 while the number of animals analyzed at P10 was increased. We observed moderate 2-3 fold upregulation of Tnf at P5 and P10 in the colon of Gfra1<sup>hypo/hypo</sup> animals (Figure 9B). Il1b mRNA expression showed increased expression in Gfra1<sup>hypo/hypo</sup> mice with remarkable inter-animal 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 may therefore reflect the end stage of the disease, which associates 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-F).

We also analyzed cytokine IFNγ, IL12, IL4, IL5, IL6, TNF, IL1b and IL9 protein levels in the serum using commercially available 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-I). We hypothesise that, systemic immune-activation with notable serum cytokine changes in Gfra<sup>hypo/hypo</sup> HSCR/HAEC mouse model is a late event which associates 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 enteric nervous system development in heterozygous Gfra1<sup>WT/KO</sup> mice grossly normal, whereas homozygous Gfra1<sup>hypo/hypo</sup> mice which have only a 20-30% further reduction in GFRα1 levels compared to Gfra1<sup>WT/KO</sup> 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 Gfra1<sup>hypo/hypo</sup> 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 to the developing kidneys. GDNF and GFRα1 can also promote synapse formation in a RET independent manner via a process called ligand-induced cell adhesion which may also influence cell migration [48], and so synapse formation could be another process where a reduction in GFRα1 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 in order to discriminate between RET-dependent from RET-independent effects in ENS and kidney development.

Our Gfra1<sup>hypo/hypo</sup> mouse provides the first opportunity to model childhood HSCR and HAEC arising from a defect in the GDNF/GFRα1/RET signaling axis. At 10 days of age, Gfra1<sup>hypo/hypo</sup> 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 humans. Importantly, although the presence of most of these pathological changes varied among the Gfra1\textsuperscript{hypo/hypo} mouse colon samples at P4 and P10, 100% of the Gfra1\textsuperscript{hypo/hypo} mice at both ages had a shift from acidic to neutral mucin production. By P10 100% of Gfra1\textsuperscript{hypo/hypo} mice also displayed similar levels of goblet cell hyperplasia, while 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 pathological changes, and that bacterial enterocyte adherence is not an initiating factor in HAEC. Gfra1\textsuperscript{hypo/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 GFRα1 in the mesenchyme of the whole fetal intestine. We found that in the duodenum of Gfra1\textsuperscript{hypo/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 Gfra1\textsuperscript{hypo/hypo} mice studied exhibited 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 only present in two of the three 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 towards neutral mucin production in the early stage followed by hyperplasia and a continuous mucin shift, and mucin overproduction during the later stages [5]. Over time, these features lead to mucus retention and crypt dilation, 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 pathological changes facilitate bacterial enterocyte adherence, bacterial invasion, and—ultimately—sepsis [6, 7]. Thus, 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 5% of all HSCR cases [1]. Various animal models of HSCR with defective endothelin signaling have been generated, and substantial progress has been made using those models towards understanding short-segment HSCR and HAEC. A careful comparison between these endothelin-defective models and our Gfra1<sup>hypo/hypo</sup> mice is an important future objective. Such a study would likely reveal similarities—and probably also differences—between the two genotypes and might pave the way for the development of gene-specific treatment strategies.

To date, more than ten HSCR susceptibility genes have been identified [1, 2, 9, 11, 49]. However, together these genes account for approximately 50% of all HSCR cases [1, 9, 49]. Thus, other genetic mutations and/or traits likely underlie HSCR. At present, neither GFRa1 protein nor GFRa1 mRNA levels are routinely measured in colon samples resected from HSCR
patients. One study using semi-quantitative PCR reported reduced levels of \( GFRal \) mRNA in three out of 13 HSCR patients analyzed [33]. Given that our results show that reducing GFRa1 levels is sufficient to cause HSCR in mice, measuring \( GFRal \) 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

Gfra1 hypomorphic (Gfra1\textsuperscript{hypo}) mice (registered in Mouse Genome Database as 129Ola/ICR/C57BL6-Gfra1\textsuperscript{tm1Joao} mouse line) were generated by inserting a pu\Delta tk cassette [31] into intron 6 of the Gfra1 gene using gene targeting via homologous recombination in embryonic stem (ES) (Figure 1A-C). ES cell clones that had undergone homologous recombination in one 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 (F) 5’-cattggccaggtgaaagaca, Gfra1loxR (R) 5’-agaaagagagatgatcacagtacatg). The mice were maintained on a 129Ola/ICR/C57BL6 mixed genetic background, housed under a 12h/12h light/dark cycle at 20-22°C, with one 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 the histological 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 (PFA) in phosphate-buffered saline (PBS) for 24 hours at room temperature (RT). Automated dehydration of the tissues and subsequent paraffin embedding were performed using an ASP300 S tissue processor (Leica). Paraffin blocks were
cut to prepare 5-µm sections using a Tissue-Tek microtome (Sakura). For whole-mount histology of P10 and P18-P25 tissues, the mice were perfused intracardially with PBS and 4% PFA. The longitudinal muscle/myenteric plexus (LMMP) preparations were isolated under dissection microscope by peeling off the outer muscle layer of the GI tract and post-fixed for 15 minutes in PFA.

**Histopathology**

Paraffin sections of the duodenum and colon were stained with hematoxylin and eosin (H&E), Gram, or a combination of Alcian blue (AB, pH 2.5) and periodic acid–Schiff (PAS) using standard protocols. LMMPs from the small and large intestine were stained for acetylcholinesterase (AChE) as previously described [50]. Briefly: LMMP was washed in PBS for 1 hour, 100 mM maleic acid buffer pH 6.0 for 5 minutes, pre-incubated 30 minutes in 65 mM maleic acid buffer including 0.5 M potassium ferricyanide, 4 mM copper sulphate and 5 mM sodium citrate and reacted 30-45 minutes in 5 mM acetylthiocholine iodide in 100 mM maleic acid buffer. NADPH diaphorase was used to visualize nitrergic neurons in LMMPs, briefly: LMMPs were washed two times 1 hour in PBS and reacted 1 hour +37ºC in a solution prepared right before use containing 0.3% Triton-X-100, 0.01% nitroblue tetrazolium chloride, and 0.1% NADPH in PBS. Finally the LMMP preparations were washed 6 times 5 minutes in PBS and mounted in glycerol for imaging.

**Grading of histopathology**

The criteria for histopathological grading are defined below. HAEC-associated inflammatory cell infiltration of the crypts (cryptitis and crypt abscesses) is milder in mice than in humans, whereas
epithelial pathology characteristic of HAEC is recapitulated well in mice. Therefore, we modified the grading system reported by Teitelbaum et al.\cite{7} to primarily reflect epithelial pathology.

**Surface epithelial damage**

Epithelial damage was graded as minimal, mild, moderate, and marked.

**Minimal surface epithelial damage** consists of mild disorganization and slight degeneration of the surface epithelium. In contrast to regular palisading, colonocytes are multifocally clustered 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 lumen of the intestine contains copious amounts of mucus and deeply eosinophilic debris of shed epithelial cells. The surface epithelium has multifocal to diffuse moderate degeneration and cuboidal to flattened cells with indistinct cell borders 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, or 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 one mouse; however, in accordance with a previous report on HAEC in mice [44], inflammatory activity is generally very low.

Marked surface epithelial damage. The surface epithelium has generally diffuse degeneration, and multiple 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 exhibits 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 leucocytes, 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 are also affected, and most of the crypts are affected.

Leukocyte numbers in the mucosa

A three-tier system was used to grade leukocyte numbers as minimal to mild (+), moderate (++) or marked (+++).

Lymphocytes

Lymphocyte number is considered to be normal if five 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 inter-cryptal 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 [44], none of our samples contained moderate or...
marked increases in lymphocytes. Accordingly, extremely few plasma cells were detected, and plasma cells were therefore excluded from grading.

Granulocytes

Eosinophils and neutrophils were graded as a single entity. Neutrophils related to surface epithelial damage (e.g., 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 are typically 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 the goblet cells

Goblet cells were counted in the distal third of the colon from the basal half of the crypts. 20-30 crypts were counted per sample. Goblet cells that were primarily 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 mM sodium citrate, 0.05% Tween 20, pH 6.0). Endogenous peroxidase activity was quenched in 1:53 H$_2$O$_2$ standard solution in Tris-buffered saline (TBS) for 30 minutes at RT. After washing with TBS containing 0.1% Tween 20 (TBS-T), the tissue was blocked in 1.5% normal goat serum in TBS-T for 30 minutes at RT. The sections were incubated overnight at 4°C in primary antibody solution containing rabbit anti-PGP9.5 (1:250; BML-PG9500, Enzo Life Sciences) or rabbit anti-GFAP (1:200; Ab-4 RB-087-A, Thermo-Scientific). After washing, the sections were incubated for 90 minutes at RT 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. 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 IFNg, IL12, IL4, IL5, IL6, TNF, EPX060-20831-90, IL-1b Mouse ProcartaPlex™ Simplex Kit, EPX01A-26002-901, and IL-9 Mouse ProcartaPlex™ Simplex Kit, EPX01A-26041-901).
qPCR

RNA isolation

RNA was isolated from snap-frozen tissues using 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 cDNA was synthesized from 50–300 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 were 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 mM dNTPs, 0.5 µl of RNase inhibitor, and 0.5 µl of Transcriptor reverse transcriptase 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 qPCR.

Quantitative real-time PCR

Quantitative PCR was performed using the LightCycler 480 real-time PCR system (Roche Diagnostics) with LightCycler 480 SYBR Green I Master mix and 2.5 pmol of primers at a final volume of 10 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, 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 two acquisitions per degree Celsius; 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. *Beta-actin* (*Actb*) was amplified as a reference gene. The following primer sets were used in this study:

- **mActb**: 5’-ctaggcgacacaagttgaaaag 5’-accagagccatacagggaca
- **mGdnf ex2-3**: 5’-cgttgacagctgaactctaatg 5’-tgccgctgtttactgtgtgacc
- **mGfra1**: 5’-ttccccacacagtttacca 5’-gcggatacattggatttca
- **mIfng**: 5’-ttcttcagcaacagcaacagge 5’-tcagcagcgaacctctttccc
- **mIl1B**: 5’-agtggacggccaccaaaag 5’-agctggatgtctctcacagg
- **mIl1a**: 5’-gtgagccaaagaaatcaagatg 5’-gtctccttttactgaacag
- **mIl1Ra**: 5’-aaccagctattgctggatcctta 5’-gcggcagaacacacattgaaggtc
- **mIl2**: 5’-tttgctttgctacagc 5’-ctgggagttcagttctctt
- **mIl4**: 5’-aaccagctacagcagagaagg 5’-tcggcagctcactgagaaca
- **mIl5**: 5’-accagcgtctgtggactaag 5’-tcctgaccaactctctttt
- **mIl6**: 5’-accacttcacaagtggagg 5’-ttgaagctgcatctgtgtg
- **mIl10**: 5’-ataaactgcacccacttcca 5’-ctgggaaccaacactttaacc
- **mIl13**: 5’-gcagcatgtgatgggttgtg 5’-tgccgaaacagttgctttt
- **mIl23**: 5’-gctgctgtgtgcttggagtctg 5’-tcggcactctctgtgtgtg
- **mRet t2**: 5’-tcctctcctacatggattga 5’-atcggccttcgtgagttgtga
- **mTgfb1**: 5’-tgagcaacagcaggtgac 5’-gtcagcagccgagtttca
**Western blot analysis**

Snap-frozen E12.5 tissue was homogenized in a homogenization solution containing 0.3 M sucrose, 10 mM HEPES, 1 mM EDTA (pH 7.2–7.4) and a proteinase inhibitor (Complete Mini-Tabs Cocktail Set, Roche); 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% SDS-page gel and transferred to a nitrocellulose membrane (Amersham Protran, GE Healthcare). After washing and blocking at RT for 60 minutes in 5% milk, the membranes were incubated in goat anti-Gfra1 antibody (1:1000; GT15004, Neuromics). After washing, the membranes were incubated at RT 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 were then stripped and re-probed 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 Image J software and an unpaired T-test was performed as a statistical analysis using GraphPad Prism software.

**Luciferase assay**

RET-dependent activation of mitogen-activated protein kinase (MAPK) by soluble GFRα1 was measured using MG87 fibroblasts that stably express RET (MG87RET) and the PathDetect-Elk1 system (#219005, Stratagene). One day prior to the assay, cells were plated in a 96-well plate (20,000 cells/well) in 100 µl/well DMEM containing 10% fetal bovine serum and 100 µg/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 were then 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 min. Luciferase activity was measured using a MicroBeta 2 plate counter. Each experiment was performed twice, and the results were analyzed using GraphPad Prism software.
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References


Reduced Gfra1 causes HSCR and HAEC in mice
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Figure Legends

Figure 1. Generation of GFRα1 hypomorphic mice and analysis of Gfra1 expression.

(A) The Gfra1 hypomorphic allele was generated by inserting a puΔtk cassette [31] into intron 6 in the Gfra1 gene. (B) Representative Southern blot analysis of EcoRV-digested genomic DNA isolated from ES 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, Gfra1\textsuperscript{puΔtk/puΔtk} 100 bp). (D) Gfra1\textsuperscript{hypo/hypo} mice develop megacolon, constipation, and abdominal distention, and they fail to thrive (note that the Gfra1\textsuperscript{hypo/hypo} mouse is smaller than the WT mouse). The point of dilation is indicated by black arrows. At P18-P25, 50% of Gfra1\textsuperscript{hypo/hypo} mice sacrificed due to obvious discomfort 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 using qPCR (N=4). (F-G) Western blot analysis of GFRα1 protein in the gut segment spanning from the duodenum to the ileum in E12.5 embryos. The panel F is a representative western blot (samples obtained from E12.5 Gfra1 KO mice were included as a negative control); the panel G shows the normalized average GFRα1 intensity (N=3 independent experiments). (H) At P10, the kidneys in Gfra1\textsuperscript{hypo/hypo} mice appear anatomically normal. (I) Gfra1\textsuperscript{hypo/hypo} mice are smaller than WT and heterozygous littermates at P10 (P<.001). The slight reduction in kidney size in the Gfra1\textsuperscript{hypo/hypo} mice likely reflects an overall failure of the mice to thrive. (J) Compared to WT littermates, kidney histology is grossly normal in P18 Gfra1\textsuperscript{hypo/hypo} mice. F, forward primer; R, reverse primer; EV, EcoRV site; ex 6, exon 6; puΔtk/puΔtk, Gfra1\textsuperscript{puΔtk/puΔtk}; KO/KO, Gfra1\textsuperscript{KO/KO}; CHO.
Reduced Gfra1 causes HSCR and HAEC in mice

Chinese hamster ovary cells transfected with a plasmid encoding GFP (-) or Gfra1 (+). In this and all subsequent figures, all summary data are presented as the mean ± SEM. *, **, and *** indicate P<.05, P<.01, and P<.001 respectively; except where indicated otherwise, statistical analyses were performed using a Student’s t-test, unless indicated otherwise.

**Figure 2. Impaired enteric neuronal progenitor colonization and hypertrophic nerve fibers in the distal gut of GFRa1 hypomorphic mice.**

(A) Immunohistochemistry using the pan-neuronal marker PGP9.5 in E13.5 WT and Gfra1<sup>hypo/hypo</sup> mice whole-mount GI tract preparations. Note the reduced numbers of neuronal progenitors (white arrows) beginning at the mid-jejunum of Gfra1<sup>hypo/hypo</sup> mice and the absence of neuronal progenitors from the distal ileum onwards; representative images are shown (N=3-4 animals per genotype). (B) Dose-dependent activation of MAPK signaling by soluble GFRa1. MG87 fibroblasts stably expressing RET and the PathDetect Elk-1 luciferase system were treated with the indicated concentration of soluble GFRa1 (N=4 biological replicates per experiment; N=2 experiments). (C-D) PGP9.5 immunohistochemistry was performed in E18.5 WT and Gfra1<sup>hypo/hypo</sup> mice from whole-mount GI tract preparations, showing reduced and scattered innervation in the mid-ileum, 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) PGP9.5 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 Gfra1<sup>hypo/hypo</sup> mice; representative images are shown.
Reduced Gfra1 causes HSCR and HAEC in mice (N=2-3 animals per genotype). The ENCC colonization of the mouse hindgut is complete by E14.5. Representative image of sagittal gut sections immunostained with the pan-neuronal marker PGP9.5 in E15.5 WT and Gfra1&lt;sub&gt;hypo/hypo&lt;/sub&gt; 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 Gfra1&lt;sub&gt;hypo/hypo&lt;/sub&gt; 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 Gfra1&lt;sub&gt;hypo/hypo&lt;/sub&gt; mice are shown.

**Figure 3. Analysis of the enteric nervous system in P10 Gfra1 hypomorphic mice.**

(A) Representative images of the pan-neuronal marker PGP9.5 immunohistochemistry in coronal gut sections prepared from P10 mice. (B) Quantification of the ganglionated plexus area (black arrowheads in A) in P10 WT and Gfra1&lt;sub&gt;hypo/hypo&lt;/sub&gt; coronal gut sections; the residual staining in the Gfra1&lt;sub&gt;hypo/hypo&lt;/sub&gt; mice colon reflects hypertrophic fibers or transition zone (white arrowheads in A). P<.05 Gfra1&lt;sub&gt;WT/hypo&lt;/sub&gt; versus Gfra1&lt;sub&gt;hypo/hypo&lt;/sub&gt; in ileum and P<.01 WT and Gfra1&lt;sub&gt;WT/hypo&lt;/sub&gt; versus Gfra1&lt;sub&gt;hypo/hypo&lt;/sub&gt; in colon, N=4-5 mice per genotype. ANOVA and Tukey’s multiple comparisons tests were used for statistical analysis. (C) Immunostaining for the glial marker, glial fibrillary acidic protein (GFAP) in the duodenum of P10 mice is similar between genotypes (N=3; representative images are shown). Black arrowheads are pointing ganglia stained with GFAP, white arrowheads marking residual staining from hypertrophic fibers of transition zone. Reminiscent of the results obtained using PGP9.5 at the same age (A-B) in the colon, Gfra1&lt;sub&gt;hypo/hypo&lt;/sub&gt; mice have a smaller GFAP-positive plexus area in the myenteric plexus relative to Reduced Gfra1 causes HSCR and HAEC in mice.
littermate controls quantified in (D); N=4-5 animals per genotype; P<.001, ANOVA and Tukey’s multiple comparisons test.

Figure 4. The hypertrophic nerve fibers in the distal gut of GFRα1 hypomorph mice are cholinergic.

(A) Acetylcholinesterase (AChE) histochemistry at P10 showing similar innervation in the duodenum, as well as cholinergic hypertrophic nerve fibers characteristic of HSCR in the colon of Gfra1<sup>hypo/hypo</sup> mice. In the ileum, an image distal of the point of dilatation is shown (N=3 animals per genotype). (B) Quantification of the AChE-positive plexus area in P10 WT and Gfra1<sup>hypo/hypo</sup> mice (N=3 animals per genotype, *** indicates P<.001). (C) NADPH diaphorase histochemistry for NOS-positive neurons in P10 mice showing similar innervation in the duodenum and an absence of NOS-positive innervation in the colon of Gfra1<sup>hypo/hypo</sup> 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 Gfra1<sup>hypo/hypo</sup> mice; N=4-5 animals per genotype (N=2 animals per genotype for the colon). ANOVA and Tukey’s multiple comparisons tests were used for statistical analysis; n.a. – not applicable for Gfra1<sup>hypo/hypo</sup> mice.

Figure 5. Histopathology of colon E18.5 and P4.

(A-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-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 arrow head) and more neutral mucins in Reduced Gfra1 causes HSCR and HAEC in mice.
Reduced Gfra1 causes HSCR and HAEC in mice

Gfra1\textsuperscript{hypo/hypo} animals (white arrow heads) quantified in F, ** indicates \(P<.01\) (N=3-4). (E) The number of goblet cells (white arrows C3, D3) is not changed at P4 (N=4-5).

**Figure 6** Mild epithelial damage, mucin retention, mucin type changes, and goblet cell hyperplasia in P10 Gfra1\textsuperscript{hypo/hypo} mice. 

(A1-2) H&E and AB-PAS (A3) staining of coronal colon sections from P10 WT mice. In WT mice, the mucosa is thin and compact with an evenly palisading, regular surface epithelium. The crypts of Lieberkühn (white arrow in A1 and A3) in the colon are shallow and straight. Goblet cells (white arrowhead in A1-3) are abundant in surface epithelium and within the proximal third of the crypts, but less abundant at the crypt bases (A3). The goblet cells in the surface epithelium and in the proximal parts of the crypts contain PAS-positive neutral mucins, staining deep purple (white arrowhead in A3). The goblet cells in the basal parts of the crypts primarily contain AB-positive acidic droplets, staining light blue (black open arrowhead in A3). (B-C) In Gfra1\textsuperscript{hypo/hypo} mice, the colon crypts (white arrow in B1, B3, and C1) are mildly to moderately dilated, with variable levels of mucin retention (black bold arrow in B2). The surface of the mucosa in the colon of Gfra1\textsuperscript{hypo/hypo} mice is “bumpy” in appearance (compare A1 with B1), and epithelial cells grow in disorganized, undulating rows with partly overlapping nuclei (black arrow in B1). The height variation of the epithelial cells (blue bold arrow in B2), the round to oval bland nuclei (black arrowhead in B2), increased eosinophilia in the apical cytoplasm (red arrowheads in B2), and the indistinct cell borders (white open arrowhead in B2) are indicative of degeneration and mild epithelial damage. Unlike in WT mice (A3), the goblet cells in Gfra1\textsuperscript{hypo/hypo} mice are often abundant in the basal parts of the crypts (white arrowheads in C2) and show a shift towards the production of PAS-positive (i.e., neutral) mucins (compare C2 with A3). (D) Quantification of goblet cells in WT, Gfra1\textsuperscript{WT/hypo}, and Gfra1\textsuperscript{hypo/hypo} mice, for which the number of goblet cells is
increased both in proximal (P<.001 and P<.01) and distal colon (P<.001) compared to both heterozygous and WT animals; N=6-8 animals per genotype. (E) Gfra1<sup>hypo/hypo</sup> mice and Gfra1<sup>WT/hypo</sup> have fewer AB-positive cells per crypt compared to WT colon (P<.001 and P<.05, N=4-5). (F). AB-PAS staining from WT and hypomorphic duodenum at P10. (G) Quantification of goblet cells (white arrows on F) in WT, Gfra1<sup>WT/hypo</sup>, and Gfra1<sup>hypo/hypo</sup> mice in P10 duodenum (N=4 per genotype). (H) Quantification of PAS positive goblet cells, AB positive goblet cells were absent in duodenum (N=4 mice per genotype). PAS, periodic acid–Schiff; AB, Alcian blue; *, **, and *** indicate P<0.05, P<0.01, and P<0.001 respectively.

**Figure 7. Histopathological analysis of moderate and marked HSCR/HAEC in P18-P25 Gfra1<sup>hypo/hypo</sup> mice.**

(A-C) Exacerbated mucin type changes, epithelial damage, scarce inflammation in the lamina propria, and a lack of bacterial invasion in P18-P25 Gfra1<sup>hypo/hypo</sup> mice with moderate-grade HAEC. (A) In WT mice, the crypts in the colon (white arrow in A1 and A2) are relatively shallow and straight, and the surface epithelium is even and regularly palisading (curved black arrow in A1). The goblet cells in the surface epithelium and in the proximal parts of the crypts contain PAS-positive (i.e., neutral) mucins, staining deep purple (white arrowhead in A2). 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; yellow arrowhead in A2) to dark blue (goblet cells; open white arrowhead in A2). In A3, Gram-positive (blue to black) and Gram-negative (intense red) bacteria are isolated from the surface epithelium by a layer of mucus (black arrow in A3). (B) The intestinal lumens of Gfra1<sup>hypo/hypo</sup> mice contain fecal material and copious amounts of mucus with eosinophilic debris of shed epithelial cells (white bold arrows in Reduced Gfra1 causes HSCR and HAEC in mice
Reduced Gfra1 causes HSCR and HAEC in mice

1. B1-B3). The crypts are markedly dilated with retained mucin (white arrow in B1-B3). The surface epithelium has diffuse degeneration (curved black arrow in B1 and B2) and an increased number of goblet cells (white arrowhead in B1-B3). Multifocal necrotic and apoptotic colonocytes (open white arrowhead in B2 and B3) and scattered intraepithelial neutrophils (red open arrowhead in B2). (C). The basal lamina propria contains moderately increased numbers of eosinophils (black arrowhead in C1) and mildly increased numbers of lymphocytes (yellow open arrowhead in C1), indicative of an inflammatory process. Note the reduction in AB-stained (i.e., acidic) mucin (black open arrowhead in C2) in the cytoplasm of basal crypt colonocytes. Gram staining shows copious amounts of mucus (black bold arrow in C3) and a lack of bacteria in mildly dilated crypts (white arrow in C3). (D-E) Marked epithelial damage at the surface epithelium, crypt abscesses, bacterial invasion, and inflammation in P18-P25 Gfra1<sup>hypo/hypo</sup> mice with marked HAEC score. (D) In Gfra1<sup>hypo/hypo</sup> mice, crypt dilatation (white arrow in D1) along with sparse neutrophil infiltrates in basal (red open arrowheads in D1) and apical (red open arrowheads in D2) crypt epithelium. The superficial epithelium displays multifocal necrotic areas and degenerative changes accompanied by superficial dense infiltrates of neutrophils (i.e., microabscesses; green arrowheads in D2). Crypt abscesses (green arrow in E1), dense bacterial aggregates (black arrow in D3), and enterocyte-attaching bacteria (green bold arrow in D3 and E2) are also indicated. Bacteria inside a submucosal blood vessel (black arrow in E3) and a bacterial aggregate in mildly dilated crypt base (white arrow in E3) are present. Single granulocytes (yellow bold arrows in E3) and lymphocytes (yellow arrowheads in E3) are present in the lamina propria and submucosa, indicative of inflammation.

Figure 8. Histopathological analysis of HSCR/HAEC in P18-P25 Gfra1<sup>hypo/hypo</sup> mice.

Reduced Gfra1 causes HSCR and HAEC in mice
Reduced Gfra1 causes HSCR and HAEC in mice

(A) A WT colon. (B-C) Focal epithelial ulceration, variable colon wall changes after loss of mucosal integrity, and bacterial invasion in P18-P25 $Gfra1^{hypo/hypo}$ mice. Marked subepithelial edema (grey arrow in B1) and basal edema (green arrow in B1) in the lamina propria, and copious edema in the submucosa (red arrow in B1). Vacuole formation, indicative of acute degenerative changes (yellow arrow in B1 and B2) and mild dilatation of crypts in the colon (white arrow in B1) are also present. Copious numbers of bacteria are attached to the surface epithelium (green bold arrows in C1). Reactive mesothelial cells (green arrowheads in B2) and thin infiltrates of eosinophils (red arrowhead in B2), neutrophils (red open arrowheads in B2), and lymphocytes in the submucosa (yellow open arrowhead in B2) are present. Focal marked epithelial damage with epithelial degeneration and necrosis (black arrow in B3) are present. Subepithelial small hemorrhage (blue arrow in B3) and infiltrating neutrophils (red open arrowheads in B3) are present. Also evident is a relative absence of AB-PAS–positive cells (compare to panels A2 and C2 in Figure 7), which indicates goblet cell degeneration (B4).

Figure 9. 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 upregulated in P5 colon (P<.05, ANOVA and Tukey’s multiple comparisons) and in P10 colon (P<.05, ANOVA and Tukey’s multiple comparisons). (E-F) Tnf and Il1b mRNA levels are not upregulated in the small intestine (a segment spanning duodenum-jejunum was analyzed). (G-J) 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. Number of animals is given for each time
Reduced Gfra1 causes HSCR and HAEC in mice

point in the Figure panels (qPCR: 2-3 biological replicates, 1-2 independent experiments per animal, ELISA two biological replicates, one experiment per animal).
Reduced Gfra1 causes HSCR and HAEC in mice

Tables

Table 1. Distribution of offspring genotypes born from breedings between Gfra1\textsuperscript{wt/hypo} animals. At E18.5, Gfra1\textsuperscript{hypo/hypo} mice are present at the expected Mendelian ratio, but not at P10 (P<.05) or at P15-P23 (P<.001), Chi-square analysis.

Table 2. Summary of histopathological findings at P4. Abbreviations: HPF, high-power field; AB, Alcian blue; PAS, periodic acid–Schiff. See Methods for details regarding the histopathological grading.

Table 3. Summary of histopathological findings at P10. Abbreviations: HPF, high-power field; AB, Alcian blue; PAS, periodic acid–Schiff. See Methods for details regarding the histopathological grading.

Table 4. Summary of histopathological findings at P18-P25. Abbreviations: HPF, high-power field; AB, Alcian blue; PAS, periodic acid–Schiff. See Methods for details regarding histopathological grading.

[Insert tables here]
**Table 1.** Distribution of offspring genotypes born from breedings between $Gfra1^{WT/hypo}$ animals.

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*) Neutrophils relating to surface epithelial injury omitted here
### Table 3. Summary of histopathological findings at P10

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Minimal: 0-4; Mild: 5-15; Moderate: 20-30; Marked: >30

*) Neutrophils relating to surface epithelial injury omitted here
### Table 4. Summary of histopathological findings at P18-P25

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*) Neutrophils relating to surface epithelial injury omitted here
Histopathology of colon P18-P25

Normal

A

Moderate

B

C

Marked

D

E
A. Detectable cytokines colon P10

B. Tnf distal colon

C. Il1b distal colon

D. Tgfb1 distal colon

E. Tnf small intestine

F. Il1b small intestine

G. TNF serum

H. IL5 serum

I. IL6 serum