Phenotype variation in two-locus mouse models of Hirschsprung disease: Tissue-specific interaction between *Ret* and *Ednrb*

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Communicated by Leland H. Hartwell, Fred Hutchinson Cancer Research Center, Seattle, WA, December 11, 2002 (received for review August 7, 2002)

Clinical expression of Hirschsprung disease (HSCR) requires the interaction of multiple susceptibility genes. Molecular genetic analyses have revealed that interactions between mutations in the genes encoding the RET receptor tyrosine kinase and the endothelin receptor type B (EDNRB) are central to the genesis of HSCR. We have established two locus noncomplementation assays in mice, using allelic series at *Ednrb* in the context of *Ret* kinase-null heterozygotes, to understand the clinical presentation, incomplete penetrance, variation in length of aganglionic segment, and sex bias observed in human HSCR patients. Titration of *Ednrb* in the presence of half the genetic dose of *Ret* determines the presentation of an enteric phenotype in these strains, revealing or abrogating a sex bias in disease expression depending on the genotype at *Ednrb*. RET and EDNRB signaling pathways are also critical for the normal development of other tissues, including the kidneys and neural crest-derived melanocytes. Our data demonstrate that interaction between these genes is restricted to the enteric nervous system and does not affect renal, coat color, and retinal choroid development.

Complex inheritance minimizes the impact of genetic variation and suppresses the potential expression of deleterious phenotypes by requiring the combined influence of mutant alleles at multiple loci to reveal a disease phenotype. Hirschsprung disease (HSCR) or aganglionic megacolon is a complex trait requiring the interaction of multiple genes for disease expression. Mutations in eight genes have already been identified in patients with HSCR and include *RET*, *GDNF*, *NRTN*, *EDNRB*, *EDN3*, *ECE1*, *SOX10*, and *SMADIP1* (1–13). HSCR also displays several genetic hallmarks, including incomplete penetrance and pleiotropic effects of mutant genotypes, a marked sex difference in clinical expression, and variation in penetrance with extent of aganglionosis. Genetic and epigenetic modification of known mutations may provide a parsimonious explanation for these findings, suggesting that the majority of HSCR cases are likely to arise from the combined effects of multiple susceptibility genes. Although studies in mice have uncovered the disease phenotypes resulting from mutations within HSCR genes, we know little of the influence of variation in multiple genes or genetic background. Variants that abrogate a gene's capacity to moderate disease expression reveal interactions intrinsic to the associated trait. We have begun to dissect these interactions in enteric nervous system (ENS) development and HSCR and report specific genetic buffering interactions in ENS development and HSCR.

HSCR is a congenital malformation with an incidence in the general population of 1/5,000 live births (14, 15) and is characterized by an absence of neural crest (NC)-derived intrinsic ganglia along a variable length of the distal intestinal tract. The pathways mediated by the RET receptor tyrosine kinase and the G-protein-coupled endothelin receptor type B (EDNRB) are critical for the normal development of the ENS and are known to be central to the genesis of HSCR. These pathways are also pleiotropic, and their signaling during embryogenesis demonstrates marks tissue-specific sensitivity.

HSCR-like phenotypes have been reported in a wide array of higher organisms, including horse, pig, rat, and mouse (16–23). Mouse models, in particular, have been crucial to the identification and functional analysis of genes underlying HSCR. Homozygous *Ret* kinase-null mice demonstrate complete enteric aganglionosis, defects in sympathetic innervation, and bilateral renal agenesis (18). In contrast, an absence of EDNRB signaling in the embryo results in severe epidermal hypopigmentation and bilateral absence of the retinal choroid layer but is also accompanied by intestinal aganglionosis (distal gut only) (16, 17). Mutations have since been identified in the human *RET* (5, 24) and *EDNRB* genes (6, 25). These mouse strains are the current models for HSCR. However, they also demonstrate several key differences from human disease. First, intestinal aganglionosis is restricted to the homozygous null genotypes in *Ret* and *Ednrb*, yet HSCR patients frequently are heterozygous for mutations in these genes. Patients homozygous for mutations within *EDNRB* have WS4 (Waardenburg–Shah syndrome; Online Mendelian Inheritance in Man, no. 277580; www.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?277580) (26), presenting with hypopigmentation anomalies, akin to the *Ednrb* mouse models (26). Second, these mouse strains display full phenotypic penetrance in homozygotes. However, human mutations in these genes are incompletely penetrant and display significant intra- and interfamilial variation (14, 27). Third, the mouse models do not demonstrate a sex bias, whereas human mutations in the same genes result in a 2- to 4-fold predominance of male affecteds. The overlapping role of RET and EDNRB signaling in ENS development raises the question whether these pathways are functionally independent. This is doubtful, because our recent studies of HSCR in an Old Order Mennonite kindred have demonstrated interaction between alleles of *RET* and *EDNRB* (28).

These data prompted us to examine the potential for noncomplementation between these loci using extant mutations in the mouse. We have analyzed allelic series at *Ednrb* (wild-type, +; piebald, *s*; piebald lethal, s^l) in *Ret* kinase-null heterozygotes (kinase-null allele, −) in a series of two-locus noncomplementation tests to uncover genetic interactions. The hypomorphic piebald allele of *Ednrb* ($Ednrb^s$) results in a 75% reduction in the corresponding transcript in homozygotes (17), although the causative molecular variant remains to be identified. $Ednrb^s/Ednrb^s$ mice are characterized by a small hypopigmented belly spot and occasional dorsal spotting and have a low incidence of megacolon (10%) in the mouse-fancier strain in which it arose (29). The spontaneous null allele of *Ednrb* (Piebald lethal, $Ednrb^{s\text{-}l}$) results from a deletion of all coding exons (17). $Ednrb^{s\text{-}l}/Ednrb^{s\text{-}l}$ mice develop megacolon and are almost completely devoid of coat color, except for an occasional pigmented blaze on the head or rump (17, 30). $Ednrb^{s\text{-}l}/Ednrb^s$ mice are also

Abbreviations: HSCR, Hirschsprung disease; EDNRB, endothelin receptor type B; dpc, days postcoitum; AchE, acetylcholinesterase; H&E, hematoxylin/eosin; ENS, enteric nervous system; NC, neural crest; RPE, retinal pigmented epithelium.

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 www.pnas.org/cgi/doi/10.1073/pnas.0337540100

hypopigmented (40–60% white spotting) with a reported incidence of megacolon of 8.3% (1/12) at 12 mo (30). The *Ret* targeted mutation deletes a critical lysine (Lys-748) at the beginning of the tyrosine kinase domain, abolishing the kinase activity of its receptor protein product and truncating RET at this point (18). We have assumed this allele to be a genetic and functional null, consistent with the report of an identical phenotype in mice harboring another targeted *Ret* null allele, terminating the protein product at the signal peptide (31). However, it remains possible that the truncated protein retains weak dominant negative activity. Ret^{-}/Ret^{-} mice display renal agenesis or severe dysgenesis and an absence of intrinsic ganglion cells throughout their digestive tracts and die in the first 24 h postpartum (18). All of the mutations described above are complete recessives with respect to the enteric phenotype.

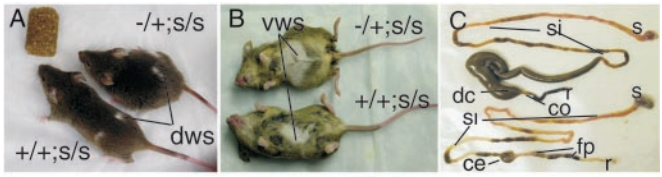
We have established oligogenic mouse strains, titrating the genetic contribution of *Ednrb* in the context of *Ret* kinase-null heterozygotes. Placing the hypomorphic $Ednrb^{s}$ allele, as a homozygote or transheterozygote with the *Ednrb* null allele ($Ednrb^{s\text{-}l}$) in combination with a *Ret* kinase-null heterozygote, results in mice presenting with a stark HSCR phenotype before weaning. Our data demonstrate that some of these oligogenic models recapitulate the sex bias observed in human HSCR, and that decreasing *Ednrb* in these mice abolishes this bias. These data indicate that the effect of mutations at *Ret* may be modulated by mutations in other HSCR susceptibility genes, and that interaction between *Ret* and *Ednrb* is a causative mechanism of HSCR (28). Furthermore, despite the fact that these pathways individually influence several aspects of development, interaction between these loci appears restricted to the ENS.

Materials and Methods

Generation of Intercross Mice. We established a series of matings titrating the genetic contribution of *Ednrb* in the context of *Ret* kinase-null heterozygotes (Ret^{-}/Ret^{+}). Ret^{-}/Ret^{+}; $Ednrb^{+}/Ednrb^{+}$ mice were mated with Ret^{+}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ mice, to assess the potential for interaction between these loci. Similarly, Ret^{-}/Ret^{+}; $Ednrb^{+}/Ednrb^{+}$ mice were mated with Ret^{+}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$ mice. Compound heterozygous offspring (Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{+}$, $n = 109$, age >12 weeks nor Ret^{-}/Ret^{+};/$Ednrb^{+}$, $n > 35$, age >12 weeks) did not demonstrate an enteric phenotype. We used progeny from these matings in a second mating series (Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{+} \times Ret^{+}/Ret^{+}$; $Ednrb^{s}/Ednrb^{s}$ and Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{+} \times Ret^{+}/Ret^{+}$; $Ednrb^{s}Ednrb^{s}$), generating 275 and 105 offspring, respectively. Details of strain maintenance, signs of distress, and the proportions of each genetic class produced by the mating programs can be found in *Supporting Text* and Table 2, which are published as supporting information on the PNAS web site, www.pnas.org. All animal studies were performed under protocols approved by the Johns Hopkins University Animal Care and Use Committee. Staining of intestines for acetylcholinesterase (AchE) activity was performed as described (28).

Quantification of Pigmented Surfaces. Digital images of the dorsal and ventral surfaces of each mouse were taken by using a Sony DSC S-70 digital camera. Values for complete dorsal and ventral surface areas and corresponding values for hypopigmented areas were determined by using NIH IMAGE software (http://rsb.info.nih.gov/nih-image), as described (32). Values for the percentage of each surface and percentage of the total surface area occupied by hypopigmented hair were then calculated (see Table 3 and Fig. 6, which are published as supporting information on the PNAS web site).

Hematoxylin/Eosin (H&E) Staining of Paraffin-Embedded Tissues. All tissues for H&E staining were fixed for 2 h at 4°C in 10% buffered formalin. Five-micrometer sections were cut from paraffin-embedded tissues and stained with H&E (standard protocols). Sections were examined by using bright-field microscopy under a Zeiss (Axiophot) photomicroscope.



D

	Ednrb					
Ret	+/+	s/+	s-l/+	s/s	s-l/s	s-l/s-l
+/+	0/100	0/84	0/35	1/155	1/60	35/35
-/+	0/50	0/109	0/35	53/53	22/22	nd
-/-	19/19	nd	nd	nd	nd	nd

Fig. 1. Oligogenic HSCR mice present with abdominal distension, demonstrating spastic distal bowel and dilatation of the proximal bowel and cecum. Dorsal surfaces (*A*), ventral surfaces (*B*), and intestinal tracts (*C*, stomach to recto-anal junction) from Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ (*Upper*) and Ret^{+}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ (*Lower*) mice are shown. (*D*) Incidence of megacolon in two locus *Ret-Ednrb* mouse models. Genotypes at *Ednrb* and *Ret* are listed left to right, upper, and in the left column, respectively (see *Supporting Text for Fig. 1*, which is published as supporting information on the PNAS web site). dws, dorsal white spotting; vws, ventral white spotting; s, stomach; si, small intestine; ce, cecum; co, colon; r, rectum; fp, fecal pellet; dc, dilated colon, +, wild-type; −, Ret^{-}; s-l, $Ednrb^{s\text{-}l}$; s, $Ednrb^{s}$; nd, not done.

***In Situ* Hybridization on Frozen Sections.** Wild-type embryos were obtained from timed matings established with CD1 mice, and 12:00 p.m. (noon) of the day that vaginal plugs were observed defined as 0.5 days postcoitum (dpc). Nonradioactive cryosection *in situ* hybridization was performed as described (33). Digoxigenin-labeled antisense probes were made with templates 0.96-kb *Ednrb* (nt 555-1514, pWP40 *Kpn*I T7 RNA polymerase), and 2.5 kb *Ret* (pmcRet7 *Not*I T7 RNA polymerase).

Statistical Analyses. Determination of statistical significance in comparisons of extent of aganglionosis and white spotting were assayed by using Student's *t* test, assuming unequal variances. Determination of statistical significance in comparisons of variances in aganglionic and white-spotting phenotypes were assayed by using an *F* test of variances. Significance was attributed for values $P < 0.05$ in either test.

Results

Noncomplementation of *Ret* Kinase-Null Heterozygotes by Mutant Alleles at *Ednrb*. Singly, the null alleles of *Ret* and *Ednrb* are strictly recessive for the enteric phenotype. The hypomorphic $Ednrb^{s}$ allele, as a heterozygote, homozygote, or transheterozygote with the *Ednrb* null allele ($Ednrb^{s\text{-}l}$), does not reduce EDNRB signaling sufficiently to result in an enteric phenotype, despite progressively stronger effects on coat pigmentation. When mutations in both *Ednrb* and *Ret* are combined, EDNRB levels in the presence of half the genetic dose of *Ret* determine the enteric phenotype. Fig. 1*D* reports the incidence of megacolon for each genotype generated in this study.

Onset and Clinical Presentation of Disease. All animals were examined daily from birth. Mice displaying abdominal distension or signs of distress (see *Supporting Text*) were identified and killed immediately (Fig. 1). Gross examination of intestinal tracts of

such mice revealed a variable length of spastic distal colon with dilated proximal colon and ileocecal junction (Fig. 1). All animals were genotyped at weaning (3–4 weeks) and killed between the ages of 4–6 weeks with the exception of three mice listed below. Among mice derived from Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{+} \times Ret^{+}/Ret^{+}$; $Ednrb^{s}/Ednrb^{s}$ backcross matings, only animals of genetic class Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ displayed abdominal distension and distress, comprising 53/275 progeny generated. Anticipating equal numbers of each genotype, this number is significantly lower than expected ($P < 0.002$, $\chi^2 = 5.48$) and is consistent with loss *in utero* or in the early postnatal period (see Table 2). A small number of animals (≈12) were unavailable for study due to infanticide or because they died before the third postnatal day and were cannibalized by their mother. Likewise, among mice derived from Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{+} \times Ret^{+}/Ret^{+}$; $Ednrb^{s}/Ednrb^{s}$ matings, 100% (22/22) of those displaying abdominal distension or distress were of the genotype Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$ and comprised 22/105. Anticipating equal numbers of each genotype, this number is not significantly lower than expected ($P < 0.33$, $\chi^2 = 0.96$) (see Table 2). The apparent increased lethality in the former cross may reflect the larger number of animals assessed ($n = 275$) compared with the latter cross ($n = 105$) or the differing genetic backgrounds of the parental strains (see *Supporting Text*).

Human HSCR patients also demonstrate a marked sex bias with a 2- to 4-fold higher incidence in males, depending on the length of the affected segment (15). Consequently, we compared the penetrance of the overt disease phenotype (abdominal distension and distress) among males and females of genotype Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$. One hundred percent of males (28/28) of this genotype were visibly affected on open field examination, compared with 68% of females (17/25). Gross examination of intestinal tracts from the remaining eight Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ females (five females killed at 4–6 weeks, one at 10 weeks, and two aged 12 weeks) also revealed a variable length of spastic distal colon. To determine the impact of further reducing the genetic contribution of *Ednrb* on presentation of disease, we compared the incidence of distension and distress among males and females of genotype Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$. Disease expression was fully penetrant in both sexes of this genotype.

Pathological Presentation of Disease. HSCR is defined by the absence of NC-derived enteric ganglia (aganglionosis) from the myenteric (Auerbach) and submucosal (Meissner) plexuses of the gut (34). We performed AchE staining of whole-mounted intestinal tracts and H&E staining of paraffin-embedded tissue sections from mice of all genotypes generated, to assess the presence or absence of enteric ganglia. The normal reticulate plexuses of intrinsic ganglia were absent from variable lengths of the intestinal tract of Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ (Fig. 2) and Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$ (data not shown) mice, progressing from the recto-anal junction (constant inferior limit) and extending proximally. The myenteric plexus was replaced by large AchE-positive extrinsic nerve fibers. The plexus was restored as fibers contacted an increasing density of ganglia, although the upper limit of normal innervation was highly heterogeneous. The hypertrophic AchE-positive neurites observed in whole-mounts of affected guts (Fig. 2) are characteristic of HSCR and contrast with the normal reticulate pattern of enteric innervation observed in age-matched Ret^{+}/Ret^{+}; $Ednrb^{+}/Ednrb^{+}$, Ret^{-}/Ret^{+}; $Ednrb^{+}/Ednrb^{+}$ (data not shown), and Ret^{+}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ controls (Fig. 2). One explanation for the observation of incomplete penetrance in Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ females is that the unaffected mice do not display intestinal aganglionosis. However, all Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ females demonstrated intestinal aganglionosis. Likewise, all Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ mice lacked ganglion cells throughout a variable length of their distal gut. Finally, the extent of aganglionosis observed in overtly unaffected versus affected Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ females was within the range of extents displayed by overtly affected females (data not shown). Thus, additional genes or effects must be necessary for clinical phenotype expression.

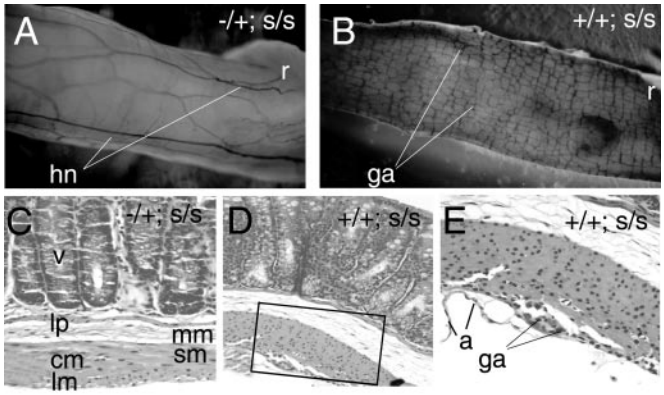


Fig. 2. Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ mice lack intrinsic ganglia along a variable length of the distal colon. (*A* and *B*) AchE-stained whole-mount intestinal tracts from oligogenic (Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$) mice (*A*) and a piebald homozygous (Ret^{+}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$) mouse (*B*). (*A*) Distal intestinal segment from a Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ mouse, demonstrating an absence of ganglia and presence of hypertrophic AchE-positive neurites. (*B*) Normal myenteric plexus in the Ret^{+}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ mouse. (*C–E*) H&E-stained paraffin-embedded sections through mouse intestines. (*C*) Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$. (*D*) Ret^{+}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ at ×20. (*E*) A ganglion cell (×40) observed (boxed) in *D* (Ret^{+}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$). +, wild-type; −, Ret^{-}; s-l, $Ednrb^{s\text{-}l}$; s, $Ednrb^{s}$; v, villus; lp, lamina propria; mm, muscularis mucosa; sm, submucosa; cm, circular muscle; lm, longitudinal muscle; a, adventitia; hn, hypertrophic neurite; ga, ganglion cell; r, recto-anal junction.

Sex Difference in Disease Severity. These observations led us to hypothesize that the length of the aganglionic segment in Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ mice may influence clinical expression and thus may differ between males and females. To determine the impact on disease expression, we assayed the extent of aganglionosis among animals of genotype Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ in whole-mount intestinal segments (recto-anal junction to cecum, inclusive) stained for AchE activity. Extent of aganglionosis was defined as the distance between the recto-anal junction and correctly innervated bowel and is reported as a percentage of the recto-anal junction to cecum measurement. The mean extent of aganglionosis in Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ male mice was 41% ($n = 26$, see Figs. 1*D* and 3), in contrast to a mean of 28% ($n = 23$) among females of the same genotype: this difference is highly significant ($P = 0.0004$). The correlation between incomplete penetrance and a lesser extent of aganglionosis in Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ females, compared with Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ males, is consistent with our hypothesis. We further hypothesized that in mice displaying complete penetrance of disease expression (Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$), extent of aganglionosis would not differ significantly between males and females and/or would exceed an unknown threshold beyond which full penetrance would be assured.

The mean extent of aganglionosis in Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$ males was 42.5% ($n = 10$) compared with 36.9% in Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$ females ($n = 12$, Figs. 1*D* and 3). Consistent with our hypothesis, this difference was not statistically significant ($P = 0.59$). However, the phenotypic variances in Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$ males and females are increased compared with their Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ counterparts (see Table 1). Consequently, although we observe an increase in the mean extent of aganglionosis in Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$

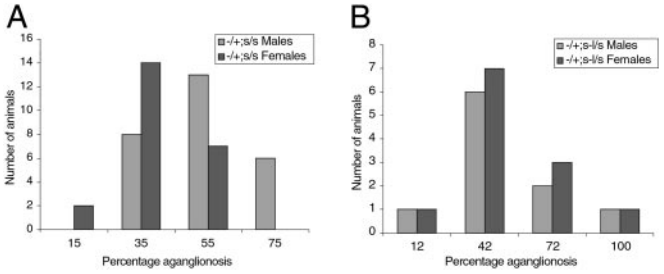


Fig. 3. *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} mice demonstrate a marked sex bias in the extent of aganglionosis. (*A*) Distribution of extents of aganglionosis illustrating the increased severity of disease observed in *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} males compared with females of the same genotype. (*B*) Distribution of extents of aganglionosis observed in *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} males and females did not differ significantly between the sexes of this genotype. Table 1 reports the *P* values for corresponding Student's *t* test and *F* test. +, wild-type; −, *Ret*$^{-}$; s-l, *Ednrb*$^{s\text{-}l}$; s, *Ednrb*^{s}.

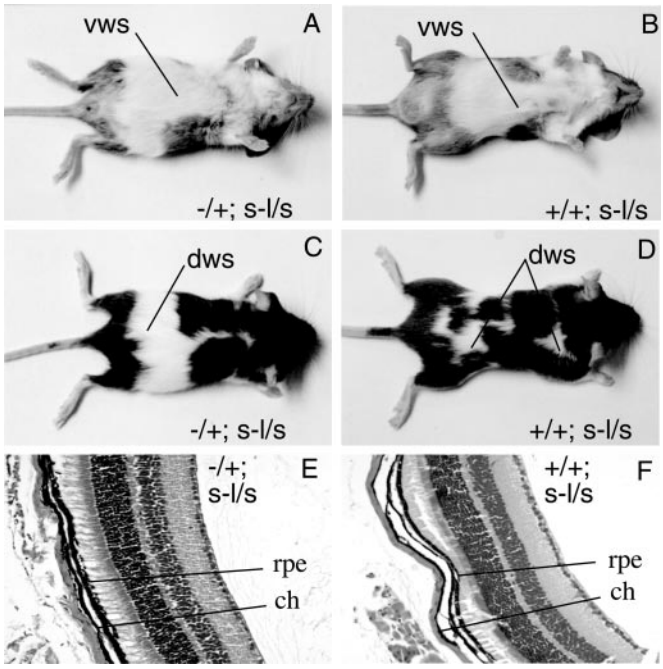


Fig. 4. Compound *Ret*/*Ednrb* genotypes exhibit no pathological impact on the development of epidermal melanocytes or the retinal choroid. Examples are shown of ventral (*A* and *B*) and dorsal (*C* and *D*) white spotting displayed by *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} (*A* and *C*), and *Ret*$^{+}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} (*B* and *D*) mice. (*E* and *F*) H&E-stained sections through the eyes of *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} and *Ret*$^{+}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s}, respectively. dws, dorsal white spotting; vws, ventral white spotting; ch, choroid; RPE, retinal pigmented epithelium.

females compared with *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} females, in the direction that is consistent with our hypothesis, the results are not significantly different ($P = 0.236$; *t* test). This may be in part due to lower statistical power. The elevated variance among *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} mice is consistent with segregation of an increased number of loci influencing the trait and may, in part, be due to presence of alleles contributed by differing strain backgrounds in *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} mice (129S1;LP/J;SSL/Le) compared with *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} (129S1;LP/J, for details, see Table 2). The increased phenotypic variance in *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} mice may also reflect the smaller sample size ascertained from this cross ($n = 12$, females; $n = 10$, males) compared with *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} mice ($n = 23$, females; $n = 26$, males).

Tissue-Specific Sensitivity to RET and EDNRB Signaling. RET and EDNRB signaling pathways are critical for the normal development of the central nervous system, peripheral nervous system, and ENS. RET is critical for development of the kidneys and EDNRB for development of NC-derived pigment cell populations, indicating a tissue-specific sensitivity to reduced levels of signaling through these pathways. The pathological influence of compound *Ret*/*Ednrb* genotypes on ENS development prompted us to determine their impact on melanocyte, retinal choroid, and kidney development.

Influence of Compound Genotypes on Melanocyte Development. Defects in EDNRB signaling result in WS4, the concurrent presentation of HSCR and Waardenburg syndrome (27). EDNRB signaling is critical for normal development of epidermal melanocytes. RET signaling also promotes epidermal pigmentation, although its role has been the subject of considerably less study (35). To determine whether heterozygosity at *Ret* (*Ret*$^{-}$/*Ret*$^{+}$) increases the degree of hypopigmentation in mice harboring *Ednrb*^{s}/*Ednrb*^{s} or *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s}, we compared the extent of white spotting between *Ret*$^{+}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} and *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} mice and between *Ret*$^{+}$/*Ret*$^{+}$;/*Ednrb*^{s} and *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} mice. Digital images of the dorsal and ventral surfaces of mice of each genotype were obtained (Fig. 4). Absolute values of percentage dorsal and ventral white spotting for animals of each genotype were calculated (see *Materials and Methods*), and their distributions are published as Table 3 and Fig. 6.

Values for dorsal spotting in *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} ($n = 18$, mean = 2.07%) and *Ret*$^{+}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} ($n = 23$, mean = 2.37%) mice were not significantly different ($P = 0.67$). Values for dorsal spotting in *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} ($n = 23$, mean = 26.89%) and *Ret*$^{+}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} ($n = 29$, mean = 24.32%) mice were also not significantly different ($P = 0.37$). Likewise, ventral spotting values were compared for the above genotype pairs and none differed significantly.

The retina contains two pigmented cell layers, the non-NC-derived retinal pigmented epithelium (RPE) and the NC-derived

Table 1. *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} animals demonstrate no sex bias in the extent of aganglionosis, in contrast to *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} mice

Sex	Genotype	Mean extent, %	Variance	Male −/+; s/s	Female −/+; s/s	Male −/+; s-l/s	Female −/+; s-l/s
Males	−/+; s/s	41.0	179.8		0.0004*	0.8787	0.5525
Females	−/+; s/s	28.2	107.2	0.1120		0.1073	0.2359
Males	−/+; s-l/s	42.5	610.8	0.0075†	0.0004		0.5892
Females	−/+; s-l/s	36.9	532.8	0.0119	0.0007†	0.4085	

P values corresponding to all comparisons of means (*t* tests) in *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} and *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} mice are reported in the upper right half of the table. Comparisons of variances (*F* tests) are reported in the lower left half of the table. +, wild-type; −, *Ret*$^{-}$; s-l, *Ednrb*$^{s\text{-}l}$; s, *Ednrb*^{s}.
*Significant difference in extent of aganglionosis between *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} males and females.
†Significant difference in variance between *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*$^{s\text{-}l}$/*Ednrb*^{s} and *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} mice.

choroid layer. EDNRB-deficient mice lack the NC-derived retinal choroid. RET is expressed in the adjacent RPE, although this layer demonstrates no defect in mice deficient in the RET ligand, glial cell line-derived neurotrophic factor (GDNF; L. Shen, personal communication). To determine the impact of the compound genotypes Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ and Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$ on the development of the choroid and RPE layers, we compared H&E-stained sections taken through the eyes of mice of the above genotypes ($n = 6$) with Ret^{+}/Ret^{+}; $Ednrb^{+}/Ednrb^{+}$ ($n = 6$) and Ret^{+}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s\text{-}l}$ ($n = 6$) mice at 4 weeks (data not shown). Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ or Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$ (Fig. 4*E*) mice develop normal pigmented choroid and RPE cell layers, identical to those observed in Ret^{+}/Ret^{+}; $Ednrb^{+}/Ednrb^{+}$, Ret^{+}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ and Ret^{+}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$ (Fig. 4*F*) mice. These findings imply that interaction between *Ret* and *Ednrb* mutations has no significant pathological impact on the development of NC-derived epidermal or retinal melanocytes.

Combined Influence of *Ret* and *Ednrb* on Renal Development. RET kinase-deficient mice (Ret^{-}/Ret^{-}) demonstrate a failure of renal induction, resulting in bilateral renal agenesis or severe renal dysgenesis (18). Although a small but significant number of *Gdnf* null heterozygotes demonstrate unilateral or bilateral renal dysgenesis (36–38), Ret^{-}/Ret^{+} mice demonstrate normal bilateral kidney development (39), suggesting that 50% of wild-type RET signaling levels may be close to the threshold required for normal renal development. EDNRB is also expressed in the Wolffian duct of the metanephros (40), although mice lacking this receptor ($Ednrb^{s\text{-}l}/Ednrb^{s\text{-}l}$) demonstrate normal renal development. We examined H&E-stained sections through kidneys from Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ and Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$ mice at 4 weeks and observed no defect in the kidney structure or organization (see Fig. 7, which is published as supporting information on the PNAS web site). These findings strongly suggest that interaction between the assayed mutant alleles of *Ret* and *Ednrb* does not significantly impact renal development.

Colocalization of *Ret* and *Ednrb* Transcripts in Migrating Enteric Neuroblasts. It is as yet unclear whether the observed impact on enteric development is the result of biochemical interaction between components of the RET and EDNRB signaling pathways or redundancy in processes contributing to a common phenotype, the formation of an intact ENS. Although RET and EDNRB are both expressed in populations of NC-derived enteric neuroblasts, their temporal and spatial expression is also tightly regulated, and no report of their colocalization exists. We performed *in situ* hybridization comparing serial sections through wild-type embryos (11.5, 12.5, and 13.5 dpc), hybridized to digoxygenin-labeled *Ret* and *Ednrb* riboprobes. Fig. 5 demonstrates that *Ret* and *Ednrb* transcripts are present in a shared subpopulation of migrating enteric neuron precursors throughout the stomach and mid-gut intestinal loops of the intestinal tract throughout 11–13.5 dpc, consistent with the hypothesis that physical interaction between these signaling mechanisms may underlie the pathological observation.

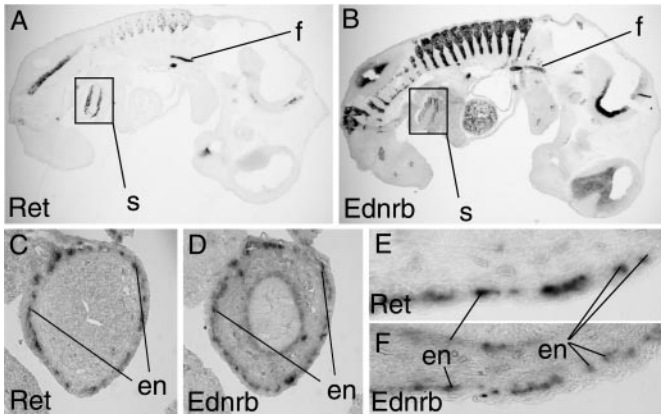


Fig. 5. Localization of *Ret* and *Ednrb* transcripts within the intestinal tract of 11.5 and 13.5 dpc embryos. (*A* and *B*) Embryos (11.5 dpc, ×25). (*C–F*) Embryonic gut (13.5 dpc, ×100). (*A*, *C*, and *E*) Migrant enteric neuroblast population in the embryonic foregut (*A*), stomach (*A*), and midgut loops (*C* and *E*) of wild-type embryos, visualized by using a digoxygenin (DIG)-labeled *Ret* antisense riboprobe on 20-μm (*A*) and 5-μm (*C* and *E*) sections. (*B*, *D*, and *F*) Sections adjacent to sections *A*, *C*, and *E*, respectively, hybridized to a DIG-labeled *Ednrb* antisense riboprobe. *Ret* and *Ednrb* riboprobes are clearly present in overlapping populations of enteric neuroblasts throughout these developmental stages. en, enteric neuron precursor; f, foregut; s, stomach.

Discussion

Interactions between *RET* and other loci, including *EDNRB*, may explain disease risk in subsets of HSCR families (25, 28, 41–44). We have used two-locus noncomplementation of existing mouse mutations in *Ret* and *Ednrb* to test these observations. We have demonstrated that certain compound genotypes of the two major HSCR genes *Ret* and *Ednrb*, which independently fail to yield intestinal aganglionosis, can result in an enteric defect presenting in the early postnatal stages. Furthermore, we report a sex bias in the penetrance of the Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ genotype. Formation of the ENS necessitates integration of a complex web of interactions, a series of checks and balances whose role it is to ensure the establishment and maintenance of a homeostatic system. As with all complex traits, the genesis of HSCR requires the interaction of molecular variants at multiple loci. These data reveal a relationship between *Ret* and *Ednrb* in buffering genetic variation in the formation of the ENS and between their mutant alleles in the genesis of an HSCR phenotype. Interaction between *Ret* and *Ednrb* mutations in these mouse strains also reveals the potential impact of other factors, e.g., the influence of gender and molecular variants at other loci, in enhancing or suppressing phenotypic variation.

Our oligogenic models display several striking characteristics. First, intestinal aganglionosis in mice combining independently benign genotypes of *Ret* and *Ednrb* requires an interaction to explain disease transmission, consistent with observations in human HSCR families. Second, mice with the compound genotype Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ demonstrate incomplete penetrance, manifest as reduced clinical expression in aganglionic females, consistent with observations in human HSCR. This may reflect gender-specific differences during development, e.g., in gene expression profile during embryogenesis and consequently in the microenvironment of the forming ENS. Third, the observed bias is gene-dosage dependent, because, once the contribution of *Ednrb* was further diminished (Ret^{-}/Ret^{+}; $Ednrb^{s\text{-}l}/Ednrb^{s}$), the sex bias is extinguished, although we did not determine the influence of further reducing RET signaling in these mice. Although our interpretation must be tempered by differences in genetic backgrounds of the intercross strains, our data suggest that sex may be revealed or masked as a HSCR susceptibility factor, depending on the magnitude of effects at the mutant loci involved. Likewise, the sex bias is diminished in HSCR patients harboring severe coding sequence mutations and presenting with the more severe long-segment HSCR (L-HSCR) (15).

The greater extent of aganglionosis observed in males of genotype Ret^{-}/Ret^{+}; $Ednrb^{s}/Ednrb^{s}$ compared with females is not reflected in the clinical literature. However, an absence of comparable data (quantitative measures of segment length and genotypes at relevant loci) means that one cannot compare the proportion of gut affected in males and females harboring equivalent mutations. Decreased penetrance in Ret^{-}/Ret^{+};

Ednrb^{s}/*Ednrb*^{s} female mice also correlates with the decreased extent of aganglionosis observed in these mice compared with *Ret*$^{-}$/*Ret*$^{+}$; *Ednrb*^{s}/*Ednrb*^{s} males. However, the observation of aganglionosis in all females failing to present with signs of distress or distension indicates that there is not a 1:1 correlation between aganglionosis and clinical presentation and suggests that our understanding of HSCR pathology is still incomplete. It is also unclear what impact these genetic lesions have on the normal function (motility, secretion, blood flow, and mucosal growth) of remaining intrinsic ganglia or their response, e.g., to endocrine factors, and how this may influence gut function.

We have demonstrated that there is no evidence for a significant impact of compound *Ret*/*Ednrb* genotypes on epidermal melanocytes or renal development, compared with either hetero- or homozygous-null *Ret* and *Ednrb* genotypes in isolation. Thus, pleiotropic activities of these genes are sensitive to interaction with other pathways in a cell-type-dependent manner.

It is not unexpected that mutant alleles of genes involved in the same biochemical system can fail to complement each other (intrinsic noncomplementation) (45, 46). Somewhat less common is the observation that mutant alleles of genes involved in biochemically distinct pathways fail to complement each other (extrinsic noncomplementation) in the genesis of a common phenotype. Intrinsic noncomplementation is common in HSCR, because the ligands of *RET* and *EDNRB* are also susceptibility genes. Our data support multiple hypotheses, including the model of intrinsic noncomplementation. Biochemical crosstalk between the G-protein-coupled receptor EPO-R and the receptor tyrosine kinase c-KIT (47) provides a clear precedent for interaction between analogous pathways, suggesting that interaction between RET and EDNRB may potentially define a novel intrinsic complementation mechanism. The tissue-specific nature of this interaction suggests that the cell type in which one chooses to address questions of biochemical interaction between RET and EDNRB is thus critically important.

We thank the current members of the Chakravarti lab for helpful discussions and comments on this manuscript and V. Pachnis and W. J. Pavan for cDNA fragments used to generate riboprobes for *Ret* and *Ednrb*, respectively. This work is supported by National Institutes of Health Grant HD28088 (to A.C.).

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