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Enteric Nervous System Progenitors Are Coordinately Controlled by the G Protein-Coupled Receptor EDNRB and the Receptor Tyrosine Kinase RET

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Summary

The enteric nervous system (ENS) in vertebrates is derived mainly from vagal neural crest cells that enter the foregut and colonize the entire wall of the gastrointestinal tract. Failure to completely colonize the gut results in the absence of enteric ganglia (Hirschsprung's disease). Two signaling systems mediated by RET and EDNRB have been identified as critical players in enteric neurogenesis. We demonstrate that interaction between these signaling pathways controls ENS development throughout the intestine. Activation of EDNRB specifically enhances the effect of RET signaling on the proliferation of uncommitted ENS progenitors. In addition, we reveal novel antagonistic roles of these pathways on the migration of ENS progenitors. Protein kinase A is a key component of the molecular mechanisms that integrate signaling by the two receptors. Our data provide strong evidence that the coordinate and balanced interaction between receptor tyrosine kinases and G protein-coupled receptors controls the development of the nervous system in mammals.

Introduction

The mammalian enteric nervous system (ENS) is composed of a large number of neurons and glia that are organized into enteric ganglia distributed throughout the gut wall (Gershon et al., 1994). The majority of ENS progenitors in vertebrates originate during embryogenesis at the vagal neural crest, which forms at the level of somites 1–7 (Le Douarin and Kalcheim, 1999). Upon delamination from the neural tube, ENS progenitors migrate ventrally to a position immediately posterior to the branchial arches and in close association with the dorsal aorta (referred to as pre-enteric neural crest cells [PENCCs]) (Durbec et al., 1996). From this region, PENCCs invade the foregut mesenchyme (called thereafter enteric neural crest cells [ENCCs]) and migrate in a rostrocaudal direction to colonize the entire length of the gastrointestinal (GI) tract (Durbec et al., 1996; Kapur et al., 1992; Natarajan et al., 2002; Young et al., 1998). In mouse embryos, PENCCs invade the foregut at E9.0–E9.5; by E10.5 ENCCs have reached the midgut loop, and by E11.5 they have colonized the entire small intestine (SI) up to the cecum, the most proximal part of the large intestine (LI) (Durbec et al., 1996; Natarajan et al., 2002). Colonization of the hindgut is completed by E13.5 (Durbec et al., 1996; Kapur et al., 1992; Young et al., 1998). Failure by ENCCs to colonize the complete gut results in the absence of enteric ganglia from variable lengths of the colon (colonic aganglionosis), the most common cause of congenital intestinal obstruction in humans (Hirschsprung's disease [HSCR]) (Chakravarti, 2001).

Two signaling pathways, mediated by the receptor tyrosine kinase (RTK) RET and the G protein-coupled receptor (GPCR) endothelin receptor B (EDNRB), have been identified independently as being critical for enteric neurogenesis in mammals (Chakravarti, 2001; Taraviras and Pachnis, 1999). RET is the main signaling component of cell surface multisubunit receptors for glial cell line-derived neurotrophic factor (GDNF) and the other members of the GDNF family of ligands (GFLs), neurturin, artemin, and persephin (Baloh et al., 2000; Saarma, 2000). The interaction between GFLs and RET is mediated by glycosyl-phosphatidyl-inositol (GPI)-linked cell surface glycoproteins, called GFRα1-4 (Airaksinen et al., 1999; Baloh et al., 2000). Mutations in *c-RET* account for approximately 50% of HSCR cases in the general population (Chakravarti, 2001), and mice with null mutations in either *c-Ret* (*Ret*$^{k-}$), *Gfrα1*, or *Gdnf* have complete intestinal aganglionosis (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). Recently, we generated two monoisoformic alleles of the murine *c-Ret* locus, *miRet*^{9} (designated hereafter *Ret*^{9}) and *miRet*^{51} (*Ret*^{51}), which express only one of the two main RET isoforms, RET9 and RET51, respectively. While RET9 alone supports normal embryonic and postnatal development, *Ret*^{51} homozygous mice are characterized by distal colonic aganglionosis, which is analogous to that observed in HSCR patients (de Graaff et al., 2001).

EDNRB is a 7-transmembrane receptor for the 21 amino acid peptides endothelin-1 (ET-1), ET-2, and ET-3 (Inoue et al., 1989). Among these peptides, ET-3 appears to be the only known functional ligand of EDNRB in the gut during mouse embryogenesis (Gershon, 1999; Leibl et al., 1999). Mutations in *EDNRB* are associated with approximately 5% of HSCR cases (Chakravarti, 2001), and mice homozygous for null alleles of *Ednrb* (such as *Ednrb*$^{s-l}$) or *Et-3* (such as *Et-3*^{ls}) have aganglionosis of the distal colon (Baynash et al., 1994; Hosoda et al., 1994; Lyon et al., 1996). The restriction of aganglionosis in EDNRB- and ET-3-deficient mice to the distal colon suggests that this signaling pathway is required during relatively late stages of gut colonization by neural crest cells (Leibl et al., 1999; Sidebotham et al., 2002). In support of this idea, a study using conditional alleles of *Ednrb* in mice has argued that the activity of this locus in the ENS is required between E11.5 and E12.5, a stage during which ENCCs cross the cecum and colonize the proximal colon (Shin et al., 1999). Most studies so far have focused on the mechanisms by which individual

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signaling pathways, such as those of RET and EDNRB, control the behavior of ENS progenitors. However, a recent report has shown that an interaction between the *RET* and *EDNRB* loci in humans and mice regulates ENS development in the distal colon (Carrasquillo et al., 2002). Despite these studies, a series of questions remain unanswered, including the temporal and spatial regulation of ET-3/EDNRB signaling within the gut during enteric neurogenesis; the potential requirement of ET-3/EDNRB and its interaction with RET for ENS development in prececal regions of the gut; the nature of the cellular responses controlled by RET and EDNRB; and the identity of the intracellular molecules that mediate the interaction between the two signaling pathways.

Here, we have used several approaches to examine the combined activity of the RET and EDNRB signaling pathways during enteric neurogenesis. More specifically, we have re-examined the pattern of expression of *Et-3* and *Ednrb* relative to the *Ret*-expressing ENCCs and show that *Et-3* is expressed with a dynamic pattern and in close association with the front of migration of these cells. We have also studied the effects of various combinations of Ret^{51} and $Et\text{-}3^{ls}$ alleles and demonstrate that a strong genetic interaction between the two loci underlies the development of the ENS throughout the mouse intestine. Using wild-type and mutant embryos, we show that ET-3 specifically enhances the proliferation-promoting effect of GDNF on undifferentiated ENS progenitors but inhibits its chemoattractive role on these cells. Such effects are likely to result from the modulation of protein kinase A activity. Taken together, our studies demonstrate that the coordinate and balanced interaction between RET and EDNRB signaling pathways controls the development of the mammalian ENS throughout the intestine.

Results

Expression of *Et-3* and *Ednrb* in the Developing Gastrointestinal Tract of Mouse Embryos

To define the spatial and temporal requirement of EDNRB activation and explore its potential interaction with RET during mammalian ENS development, we reexamined the expression of *Et-3* and *Ednrb* in the GI tract of mouse embryos between E9.5 and E13.5, stages that encompass the period of gut colonization by neural crest cells (Durbec et al., 1996; Kapur et al., 1992; Natarajan et al., 2002; Young et al., 1998). *Et-3* mRNA was first detected in the pancreatic anlage and the mesenchyme of the midgut and hindgut at E10.0 (Figure 1A), a stage at which ENCCs, identified by in situ hybridization with a *c-Ret*-specific riboprobe (Pachnis et al., 1993), had colonized the stomach and the proximal SI (Figure 1D). During the next 24 hours, as the front of migrating ENCCs progressed caudally (Figures 1E and 1F), *Et-3* expression was localized mainly at the cecum and the proximal colon (Figures 1B and 1C), where it remained at high levels until the end of ENCC migration (data not shown). Comparison of the *c-Ret* and *Et-3* expression patterns indicated that the front of *c-Ret*-positive ENCCs abutted the *Et-3*-expressing domain of the gut (Figure 1, compare panels A–C to D–F, respectively). Expression of *Et-3* ahead of the migrating ENCCs indicated that it was expressed in the splachnic mesenchyme (Leibl et al., 1999; Wu et al., 1999), which was further supported by a normal expression pattern of this gene in the GI tract of RET-deficient embryos (data not shown). Analysis of *Ednrb* expression in the gut of wild-type and Ret^{k-} mutant embryos at E11.0–E11.5 showed that the gene was expressed in ENCCs (Figures 1G–1L, arrows in panels H and I point to EDNRB-expressing ENCCs present in the small intestine and around the ileocecal junction, respectively). In addition to ENCCs, diffuse mesenchymal Ednrb-specific signal was also observed in the small intestine (jejunum) of Ret^{k-} embryos, which lack endogenous neural crest cells (Figures 1J and 1K). However, no signal was observed in the distal small intestine and the cecum of these mutants (Figure 1L). These findings suggest that, in addition to ENCCs, *Ednrb* is specifically expressed by the splachnic mesenchyme of the proximal small intestine but is absent from the splachnic mesenchyme of the cecum and proximal colon (see also Wu et al., 1999). Taken together, these studies show that during colonization of the SI, *Et-3* is expressed ahead of the invading *c-Ret*- and *Ednrb*-expressing neural crest cells. This raises the possibility that the ET-3/EDNRB signaling pathway, in addition to its requirement for enteric ganglia formation in the distal colon, plays an important role in the development of the ENS in prececal gut segments.

Genetic Interaction between Ret^{51} and $Et\text{-}3^{ls}$ Alleles

To further examine the role of RET and EDNRB signaling in the developing ENS, guts from $Ret^{51/51}$ and $Et\text{-}3^{ls/ls}$ mutant embryos ranging from stage E10.5 to E15.5 were labeled for *Phox2B* or TuJ1 to visualize ENCCs and their neuronal progeny, respectively (Natarajan et al., 2002; Young et al., 1999, 2003). Consistent with our previous findings, we observed a reduced number and delayed migration of ENCCs in the gut of E10.5 $Ret^{51/51}$ embryos relative to wild-type controls (Figure 2, compare panels A and D; see also Natarajan et al., 2002). However, the colonization defect in the SI of Ret^{51} homozygotes was largely corrected shortly afterwards, and by E12.5 the apparent number and distribution of *Phox2B*+ cells in this region of the gut were comparable to control embryos (Figure 2, compare E1–E2 to B1–B2). Consistent with this, by E15.5 an apparently normal neuronal network had formed throughout the SI of $Ret^{51/51}$ embryos (Figure 2F1). As in the SI, colonization of the LI was also delayed in Ret^{51} homozygotes. Thus, at E12.5 the cecum was partially colonized and only a small number of ENCCs had entered into the colon (Figure 2, compare panels E1–E2 to B1–B2). However, unlike the recovery in the SI, ENCCs in the LI failed to complete their migration and mature ganglionic plexi formed only in the proximal third of the colon (Figure 2, compare panels F3 and C3; see also de Graaff et al., 2001).

In $Et\text{-}3^{ls/ls}$ embryos, the number of *Phox2B*+ cells and the extent of migration in the SI at E10.5 were also reduced relative to wild-type controls (Figure 2, compare panels G and A). By E12.5, the front of migration of ENCCs had reached the ileocecal valve (located at the junction of SI and cecum), but the number of neural crest-derived cells throughout the SI appeared to be

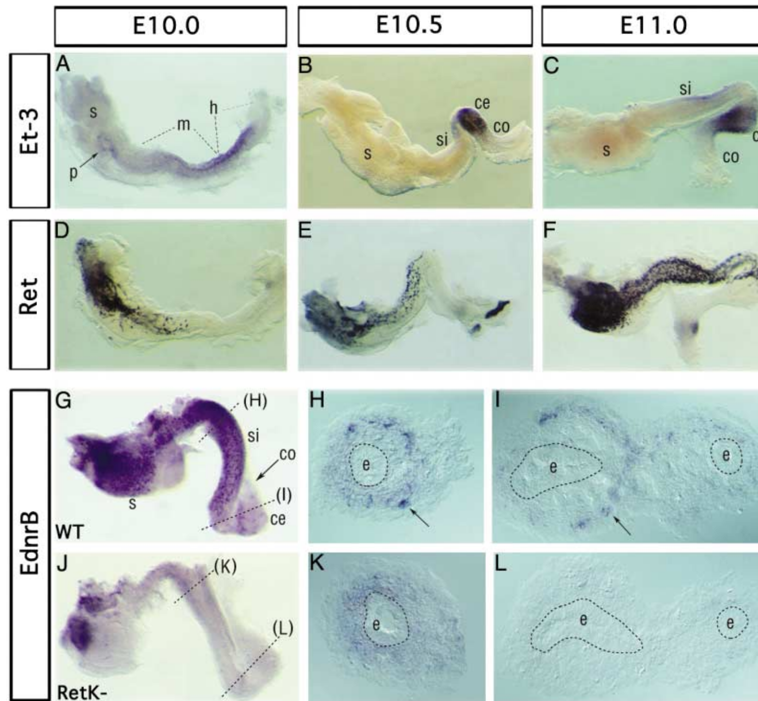

Figure 1. Expression of *Et-3* and *Ednrb* during Colonization of Mouse Fetal Gut by ENCCs

(A–F) Whole-mount in situ hybridization of E10.0 (A and D), E10.5 (B and E), and E11.0 (C and F) mouse embryonic gut with riboprobes specific for *Et-3* (A–C) and *c-Ret* (D–F). The *c-Ret*-specific signal tracks the migration of neural crest-derived cells along the rostrocaudal axis of the gut. At E10.0 (A), *Et-3* is expressed in a relatively broad segment of midgut (m) and hindgut (h), but shortly afterwards (E10.5; B) it is restricted to the cecum (ce) and the proximal colon (co). Throughout the developmental period examined here, the front of migrating ENCCs abuts the *Et-3*-expressing domain of the gut.

(G–L) In situ hybridization of E11.5 gut with an *Ednrb*-specific riboprobe. Wild-type guts were hybridized as whole-mount preparations (G), and sections corresponding to the dotted lines H (jejenum) and I (cecum and proximal colon) are shown in (H) and (I), respectively. In the jejenum (H), *Ednrb* was expressed in neural crest-derived cells (strong punctate signal) and in the splachnic mesenchyme (relatively weak and diffuse signal) distributed around the endoderm (e). In the cecum region (I), expression was observed only in ENCCs. E11.5 guts from Ret^{k-} mutants were also hybridized as whole-mount preparations with an *Ednrb* riboprobe (J). Sections through the jejenum and cecum/proximal colon are shown in (K) and (L), respectively. Both regions of the gut lack neural crest-derived signal, while mesenchyme-specific signal was observed only in the jejenum (K) and was absent from the cecum and colon regions. ce, cecum; co, colon; e, endoderm; h, hindgut; m, midgut; p, pancreas; s, stomach; and si, small intestine.

reduced relative to control guts (Figure 2; compare panels H1–H2 to B1–B2). To quantify this effect, we hybridized guts from wild-type and ET-3-deficient E12.5 embryos with a *Phox2B*-specific riboprobe and determined the number of ENCCs present on SI sections (Figures 2P and 2Q). The average number of *Phox2B*+ cells present in SI sections from ET-3-deficient embryos (39 ± 7) was significantly reduced relative to that of their wild-type littermates (69 ± 7). In addition to the reduction in cell number, no ENCCs had entered the colon at this stage (Figures 2H1 and 2H2). However, at E15.5, the number of neurons in the SI of $Et\text{-}3^{ls/ls}$ embryos was apparently similar to that of wild-type controls, but TuJ1+ cells were detected only in the proximal 1/3–1/2 of the colon (Figure 2, compare panels I1–I3 with C1–C3). In summary, these studies showed that in addition to colonic aganglionosis, both $Ret^{51/51}$ and $Et\text{-}3^{ls/ls}$ embryos have a transient defect in ENS formation in the SI (between E10.5 and E12.5), characterized by reduced number and delayed migration of ENCCs.

To examine the genetic interaction between *c-Ret* and *Et-3*, compound heterozygotes for Ret^{51} and $Et\text{-}3^{ls}$ (whose gut phenotypes were indistinguishable from wild-type controls) were intercrossed, and guts of embryos with various combinations of mutant alleles were analyzed for *Phox2B* (at E12.5) or TuJ1 (at E15.5) expression. At E12.5, in 35% of $Ret^{51/51}$; $Et\text{-}3^{+/ls}$ (n = 23) and in 70% of $Ret^{51/51}$; $Et\text{-}3^{ls/ls}$ (n = 10) embryos examined, ENCCs were virtually absent from the entire intestine and were detected only within the stomach wall (Figures 2J1, 2J2, 2L1, and 2L2). In the remaining less severely affected embryos, some ENCCs were detected in the SI, but their number and extent of migration were significantly reduced relative to $Ret^{51/51}$ guts (data not shown). This colonization defect was not corrected at later embryonic stages, as a similar proportion of double mutants at E15.5 showed drastically reduced or completely absent enteric neurons throughout the intestinal wall (Figures 2K1–2K3 and 2M1–2M3). These findings show that removal of *Et-3* activity in $Ret^{51/51}$ animals results in a phenotype (total intestinal aganglionosis) that is much more severe compared to that of ET-3-deficient or $Ret^{51/51}$ embryos (colonic aganglionosis) and suggests a strong genetic interaction between Ret^{51} and $Et\text{-}3^{ls}$ alleles.

We also compared the colonization of the gut by ENCCs between $Et\text{-}3^{ls/ls}$ and $Ret^{+/51}$; $Et\text{-}3^{ls/ls}$ embryos. At E12.5 we found that the number and distribution of *Phox2B*+ cells in the SI of the two groups were similar. However, colonization of the colon was more extensive in $Ret^{+/51}$; $Et\text{-}3^{ls/ls}$ embryos relative to single $Et\text{-}3^{ls/ls}$ mutants (Figure 2, compare N1–N2 and H1–H2). Thus, ENCCs had successfully crossed the cecum and colonized the proximal colon in 28% of $Et\text{-}3^{ls/ls}$ embryos (n = 14), but they did so in 90% of similar stage $Ret^{+/51}$; $Et\text{-}3^{ls/ls}$ embryos analyzed (n = 12). To examine whether this apparent rescue of LI colonization in E12.5 $Ret^{+/51}$; $Et\text{-}3^{ls/ls}$ embryos is maintained at later stages, we compared the distribution of TuJ1+ cells in the GI tract of $Et\text{-}3^{ls/ls}$ and $Ret^{+/51}$; $Et\text{-}3^{ls/ls}$ embryos ranging from E14.5 to E15.5. Enteric neurons were detected in the proximal LI of all embryos analyzed (Figures 2I1 and 2O1). However, and consistent with our findings at E12.5, the LI of $Ret^{+/51}$; $Et\text{-}3^{ls/ls}$ embryos was more extensively colonized by enteric neurons relative to single $Et\text{-}3^{ls/ls}$ mu-

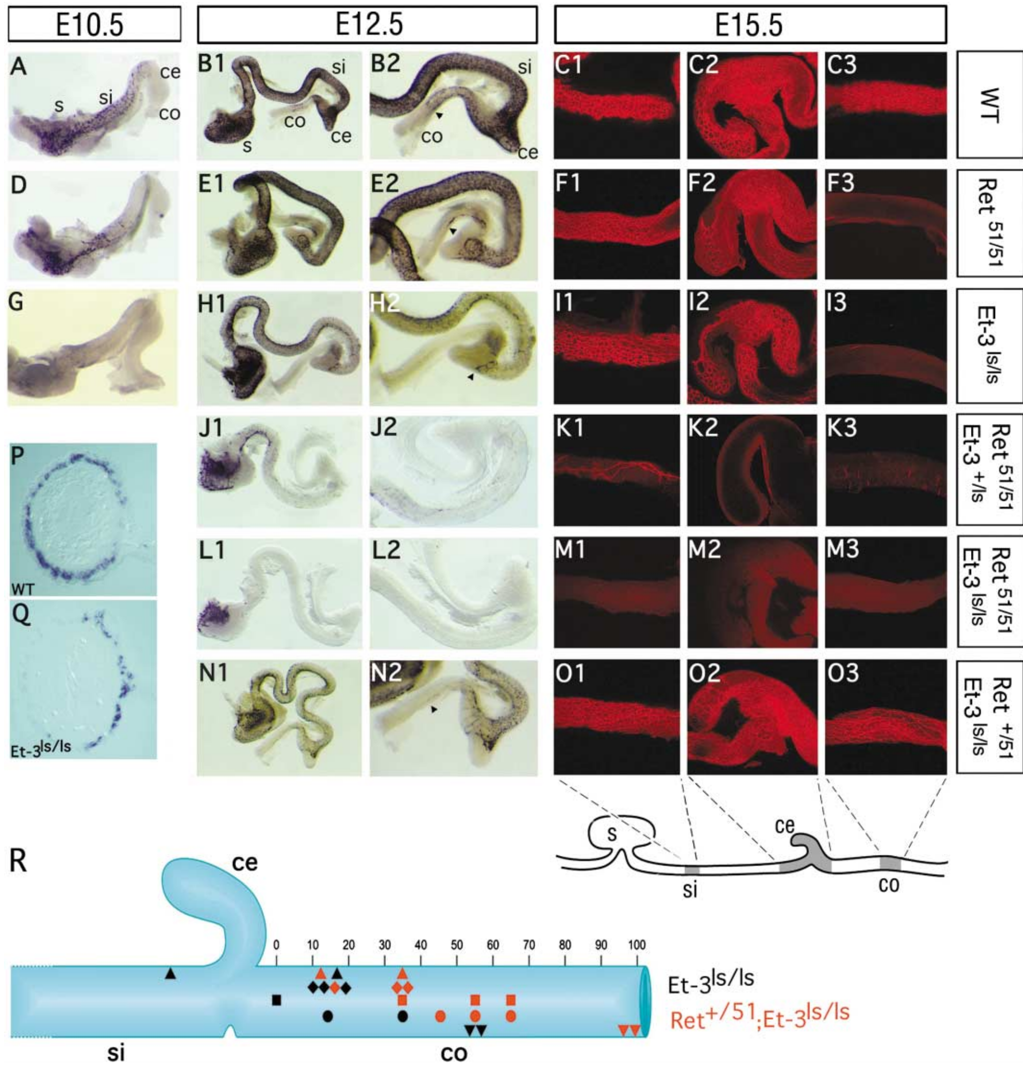

Figure 2. Genetic Interaction between *Ret*^{51} and *Et-3*^{ls} Alleles Controls the Development of the ENS throughout the Intestine

Guts were dissected from E10.5 (A, D, and G), E12.5 (B1–B2, E1–E2, H1–H2, J1–J2, L1–L2, and N1–N2), and E15.5 (C1–C3, F1–F3, I1–I3, K1–K3, M1–M3, and O1–O3) mouse embryos of the appropriate genotypes and hybridized with a *Phox2B*-specific riboprobe (E10.5 and E12.5 preparations) or stained with neuron-specific tubulin (E15.5 preparations) as whole-mount preparations. Panels B2, E2, H2, J2, L2, and N2 show enlargements of the distal small intestine, cecum, and colon of the gut preparations shown in B1, E1, H1, J1, L1, and N1, respectively. Arrowheads in B2, E2, H2, and N2 indicate the position of the front of migrating ENCCs. The diagram below the E15.5 panels is a schematic presentation of the gut. The shaded parts of the diagram show the regions of the gut represented in each column. (P) and (Q) show representative transverse sections through the small intestine of E12.5 wild-type and *Et-3*$^{ls/ls}$ mutant guts, respectively. Note the reduced number of *Phox2B*+ cells in the ET-3-deficient gut.

Combination of *Ret*^{51} and *Et-3*^{ls} alleles in the genotypes shown in panels J1–J2, K1–K3, L1–L2, and M1–M3 results in a dramatic reduction in the number of ENCCs in the entire intestine relative to the single *Ret*$^{51/51}$ (E1–E2) and *Et-3*$^{ls/ls}$ (H1–H2) mutants. However, a single *Ret*^{51} allele partially rescues the phenotype of *Et-3*$^{ls/ls}$ homozygotes at E12.5 as more *Phox2B*-expressing cells have crossed the cecum to enter the colon in *Ret*$^{+/51}$*; Et-3*$^{ls/ls}$ embryos (N1–N2) relative to stage-matched *Et-3*$^{ls/ls}$ littermates (H1–H2). This rescue effect is maintained at later stages of development as shown by the comparison of panels O3 and I3.

(R) Diagrammatic representation of distal small intestine (SI), cecum (ce), and large intestine (co) of E14.5–E15.5 embryos with numbers indicating the fraction of the colon beyond the cecum. Each symbol represents the extent of colonization of the gut by neural crest-derived cells in an individual embryo. Same shape symbols (grouped on a single line) represent littermates. Black shapes are *Et-3*$^{ls/ls}$ embryos, while red shapes represent *Ret*$^{+/51}$; *Et-3*$^{ls/ls}$ embryos. Note that the presence of single *Ret*^{51} allele generally results in more extensive colonization of the large intestine in E14.5–E15.5 *Et-3*$^{ls/ls}$ homozygous embryos. ce, cecum; co, colon; s, stomach; and si, small intestine.

tants (Figure 2, compare panels I3 and O3). This effect is shown schematically in Figure 2R, where we recorded the extent of colonization of the LI in *Et-3*$^{ls/ls}$ and *Ret*$^{+/51}$*; Et-3*$^{ls/ls}$ littermates at E14.5–E15.5. In general, the percentage length of LI covered by neuronal plexus was greater in *Ret*$^{+/51}$; *Et-3*$^{ls/ls}$ embryos relative to their *Et-3*$^{ls/ls}$ counterparts. These findings indicate that the presence of a single *Ret*^{51} allele can partially rescue the

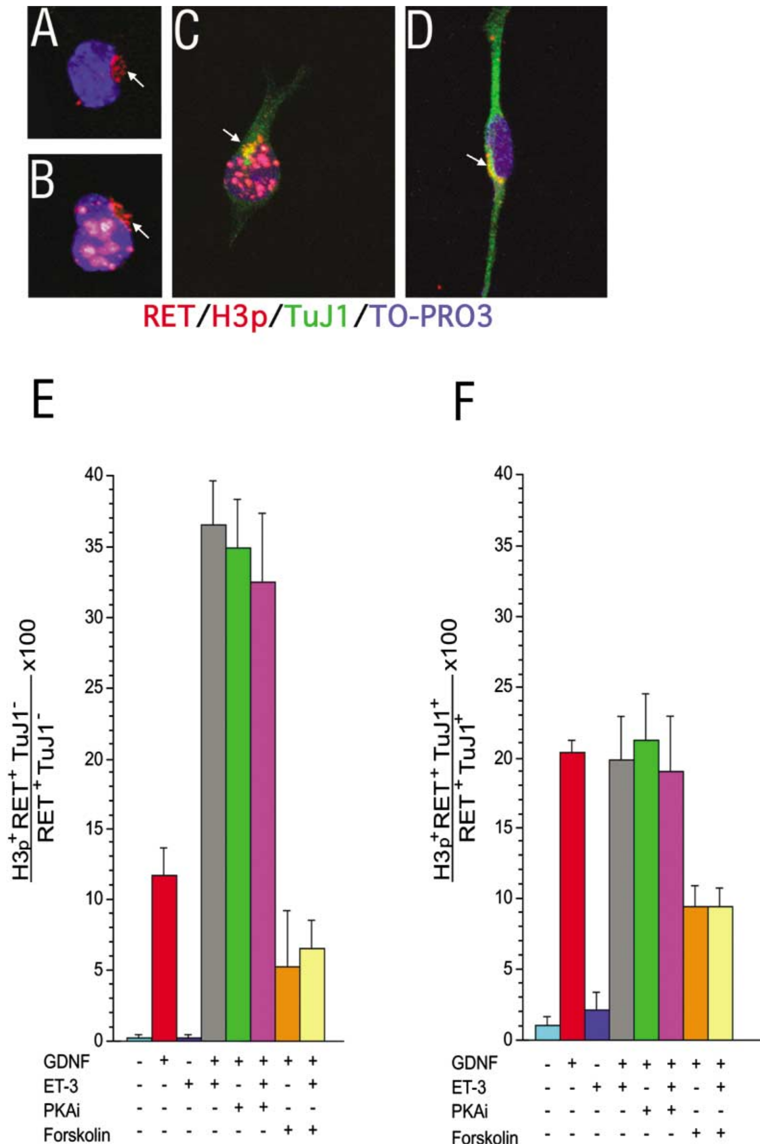

Figure 3. Synergistic Effects of ET-3 and GDNF on the Proliferation of Undifferentiated ENCCs

E11.5 mouse embryonic ceca were dissociated, cultured for up to 36 hr, and stained by immunofluorescence with antibodies for RET (red), TuJ1 (green), and H3p (red), and counterstained with the nuclear stain TO-PRO-3 (blue). Characteristic examples of individual cells from such cultures are shown in (A)–(D). In TuJ1− cells, RET expression is identified by the red cytoplasmic signal (arrows in A and B), while in TuJ1+ cells, RET expression is identified by the yellow cytoplasmic signal, which results from the superimposition of red (RET) and green (TuJ1) colors outside the nucleus (arrows in C and D). H3p+ dividing cells are identified by red signal within the nucleus (B and C). Cells in (A) and (B) are RET+/TuJ1−/H3p− and RET+/TuJ1−/H3p+, respectively. Cells in (C) and (D) are RET+/TuJ1+ ^{low}/H3p+ and RET+/TuJ1+ ^{high}/H3p−, respectively.

The histograms in (E) and (F) show the proportion of H3p-expressing undifferentiated progenitors (RET+/TuJ1−) (E) and the fraction of H3p-expressing neuroblasts (RET+ TuJ1+^{low}) (F) in cultures maintained in the indicated combinations of GDNF, ET-3, PKAi, and forskolin. Note that ET-3 on its own has relatively small effects on the percentage of proliferating cells but specifically enhances the effect of GDNF on the proliferation of undifferentiated progenitors of the ENS. The effect of PKAi is similar to that of ET-3.

aganglionosis of *Et-3$^{ls/ls}$* animals and suggest that changes in RET activity can alter the phenotypic outcome of *Et-3/Ednrb* mutations.

Synergistic Effect of ET-3 and GDNF on the Proliferation of ENS Progenitors In Vitro

The dramatic reduction in the number of ENCCs and enteric neurons in the SI of *Ret$^{51/51}$*; *Et-3$^{+/ls}$* and *Ret$^{51/51}$*; *Et-3$^{ls/ls}$* embryos relative to single *Ret$^{51/51}$* or *Et-3$^{ls/ls}$* mutants suggests that EDNRB and RET signaling together control the colonization of the fetal GI tract by ENS progenitors. Previous studies have shown that GDNF promotes the proliferation, differentiation, migration, and survival of cultured ENCCs and that ET-3 inhibits their differentiation (Focke et al., 2003; Gianino et al., 2003; Hearn et al., 1998; Heuckeroth et al., 1998; Natarajan et al., 2002; Taraviras et al., 1999; Worley et al., 2000; Wu et al., 1999; Young et al., 2001). Here, we have re-examined the effects of GDNF and ET-3, separately and in combination, on the proliferation of ENS progenitors by establishing primary cultures from the cecum of E11.0–E11.5 mouse embryos. As *Ednrb* expression was undetectable in the splachnic mesenchyme of the cecum at this stage (Figures 1I and 1L), we reasoned that any changes in proliferation of ENCCs were likely to reflect the direct effect of ET-3 on ENS progenitors. This is further supported by our failure to detect expression of *Ednrb* by RT-PCR analysis of the mesenchymal component of dissociated cecal cultures established from either wild-type or RET-deficient E11.0–E11.5 mouse embryos (data not shown). Dissociated ceca were plated at low density in the presence of various combinations of growth factors (see Experimental Procedures), and 24–36 hours after plating, cultures were analyzed for expression of RET (to identify neural crest-derived cells), TuJ1 (to identify cells committed to the neuronal lineage), and the mitotic marker phosphohistone-3 (H3p; Figures 3A–3D). This marker combination enabled us to identify the frac-

tion of dividing (H3p+) cells among the pool of undifferentiated ENCCs (RET+/TuJ1−; which constitute approximately 15% of the total RET+ population) or committed neuroblasts and neurons (RET+/TuJ1+; 85%). All dividing RET+/TuJ1+ cells expressed relatively low levels of TuJ1 and showed no signs of overt neuronal differentiation, and we never observed H3p+/RET+/TuJ1+ ^{high} cells. Relative to control medium, addition of GDNF increased significantly the fraction of H3p+ cells among both the RET+/TuJ1− and RET+/TuJ1+ cell populations (by 11.6% and 19.2%, respectively; $p < 0.001$; Figures 3E and 3F). ET-3 alone had either no effect on RET+/TuJ1− cells or resulted in a relatively small increase in the fraction of H3p+ cells of the RET+/TuJ1+ cell population (Figures 3E and 3F). GDNF and ET-3 in combination also did not change significantly the percentage of dividing neuroblasts relative to GDNF-only cultures (20.4% ± 1.2% and 19.9% ± 2.9%, respectively; Figure 3F). However, in the presence of GDNF, ET-3 led to a more than 3-fold increase in the percentage of H3p+ undifferentiated ENS progenitors (36.6% ± 3.0% versus 11.7% ± 1.8%; $p < 0.001$; Figure 3E). This effect of ET-3 was abrogated by BQ788, a specific inhibitor of EDNRB (Wu et al., 1999), indicating a requirement for activation of this receptor (data not shown). In summary, our studies show that GDNF promotes the proliferation of undifferentiated ENCCs and enteric neuroblasts and that ET-3 functions cooperatively with GDNF to specifically enhance its proliferative effect on undifferentiated ENCCs.

Inhibition of PKA Activity Mimics the Effect of ET-3 on Undifferentiated ENS Progenitors

Activation of EDNRA and EDNRB receptors can reduce the activity of cAMP-dependent Protein Kinase A (PKA) in certain cell types (James et al., 1994; Ono et al., 1994). To explore the possibility that inhibition of PKA activity mediates the effects of ET-3 on the proliferation of ENS progenitors, we examined the combined effects of GDNF and the PKA inhibitor 4-Cyano-3-methylisoquiniline (designated hereafter PKAi) (Jiao et al., 2002) on primary cultures of dissociated E11.0–E11.5 mouse embryo ceca. Similar to the effect of ET-3, addition of PKAi to GDNF-containing medium did not alter the proliferation of enteric neuroblasts (RET+/TuJ1+) but increased the percentage of H3p+ undifferentiated ENS progenitors (RET+/TuJ1−) (Figures 3E and 3F). The effect of PKAi was of similar magnitude to that of ET-3, while combination of GDNF, ET-3, and PKAi did not result in a further increase of cell proliferation in either of the two cell populations (Figures 3E and 3F). We also examined the effects of the PKA activators forskolin and db-cAMP in combination with GDNF. In the presence of either activator, the effect of GDNF on both undifferentiated ENCCs and enteric neuroblasts was reduced (Figures 3E and 3F and data not shown). Interestingly, in the presence of either compound, ET-3 failed to promote cell proliferation, indicating that this effect required inhibition of PKA activity. In a parallel series of experiments, addition of the protein kinase C inhibitor, bisindolylmaleimide (Tokuda et al., 2003), resulted in the opposite effect to that of PKAi, indicating the specificity of the PKAi compound (data not shown). Taken together, these studies show that ET-3, in conjunction with GDNF, promotes the proliferation of undifferentiated ENS progenitors and suggest that this effect requires inhibition of PKA activity.

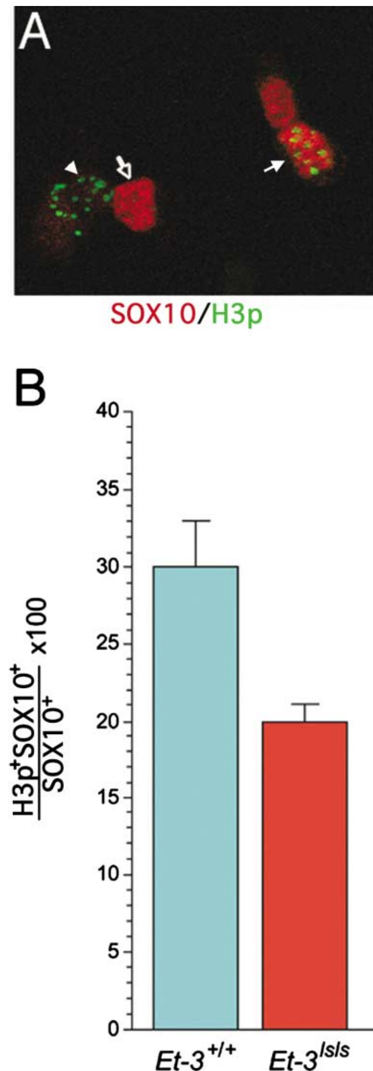

Figure 4. Reduced Proliferation of ENS Progenitors in the Gut of *Et-3*$^{ls/ls}$ Embryos

E11.5–E12.0 intestine from wild-type embryos was dissociated, cultured for up to 2 hr, and immunostained for SOX10 and H3p. Typical images of SOX10 and/or H3p expressing cells are shown in (A). Open arrow shows a SOX10+/H3p− cell, the closed arrow identifies a SOX10+/H3p+ cell, and the arrowhead shows a SOX10−/H3p+ cell.

The histogram in (B) shows the fraction of H3p+ cells within the SOX10+ population present within the cultures of wild-type (*Et-3*$^{+/+}$) and *Et-3*$^{ls/ls}$ mutants. In *Et-3*$^{ls/ls}$ mutants a smaller fraction of SOX10-expressing ENS progenitors is dividing.

Reduced Mitotic Activity of ENCCs in *Et-3*$^{ls/ls}$ Embryos

The proliferation-promoting effect of ET-3 on undifferentiated ENCCs in culture raises the possibility of a similar

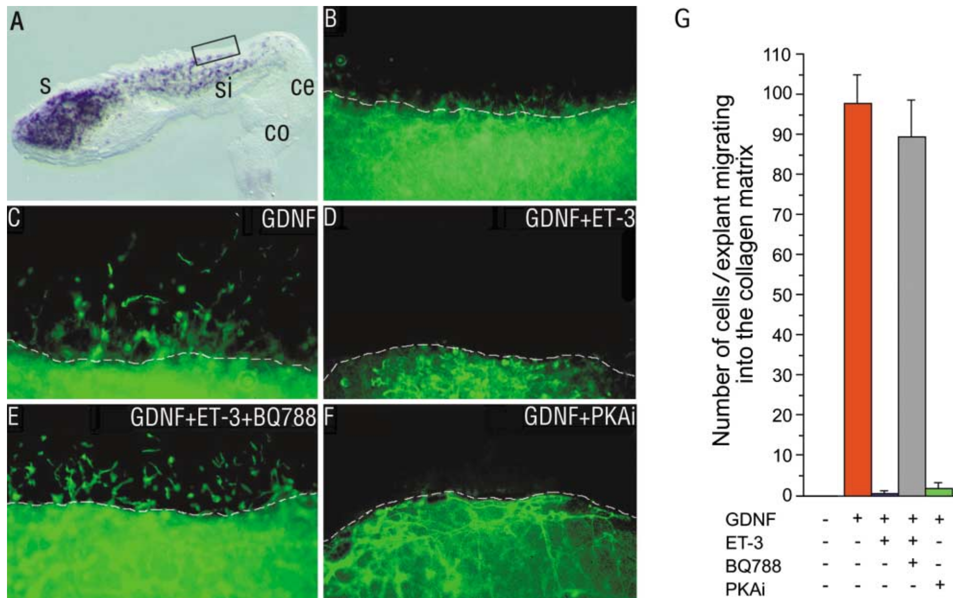

Figure 5. ET-3 Modulates the Migratory Response of ENCCs to GDNF

E10.5–E11.0 mouse embryonic gut (represented in panel A after in situ hybridization with a *Ret*-specific riboprobe to reveal ENCCs) was placed in a three-dimensional collagen gel and cultured overnight (≤16 hr) in control medium (B) or medium containing GDNF (C), GDNF+ET-3 (D), GDNF+ET-3+BQ788 (E), or GDNF+PKAi (F). At the end of the culture period, explants were immunostained for RET. ET-3 inhibits the GDNF-induced migration of ENCCs (compare panels C and D). This effect is relieved by BQ788 (E) and mimicked by PKAi (F). The box in (A) indicates the area of the gut detailed in (B)–(F). To quantify these effects, the number of RET+ cells present within the boxed area of the collagen matrix was counted in explants (n = 12) maintained under various culture conditions, and the results are presented as a histogram (G).

role for this signaling molecule in vivo. Previous studies have shown that *Sox10*, a member of the high-mobility group (HMG) gene family, is specifically expressed by neural crest stem cells (NCSCs) and undifferentiated ENS progenitors in vivo and is downregulated upon neuronal differentiation (Kim et al., 2003; Paratore et al., 2001, 2002; Young et al., 2003; and our unpublished observations). Thus, comparative analysis of *Ret* and *Sox10* expression in the gut of mouse embryos has shown that the populations of undifferentiated RET-expressing cells (RET+/TuJ1− or RET+/PGP9.5−) and SOX10+ cells are equivalent (Young et al., 2003). Furthermore, *Sox10* contributes to the maintenance of the multipotential state of NCSCs in culture (Kim et al., 2003). Therefore, using SOX10 as a marker of undifferentiated ENS progenitors, we compared the fraction of proliferating SOX10+ cells between wild-type and *Et-3*$^{ls/ls}$ mutants. For this, dissociated SI from wild-type or ET-3-deficient mouse embryos (E11.5–E12.0) were plated and analyzed for expression of H3p and SOX10 as soon as cells attached to the culture substrate (≤2 hr). The short culture period was likely to reflect the mitotic activity of gut cells in vivo. We found that 30% ± 3% of SOX10-expressing cells in wild-type cultures were dividing. However, in parallel cultures from ET-3-deficient embryos, the corresponding fraction of H3p+ cells was reduced to 20% ± 1% (p = 0.00138, Figure 4B). Analysis of the total cell population showed no difference in the percentage of dividing cells between wild-type and mutant cultures (not shown). These data are consistent with our previous findings and suggest that ET-3 is required in vivo to maintain normal levels of mitotic activity of undifferentiated ENS progenitors.

ET-3 Inhibits the Chemoattractive Effect of GDNF on ENCCs

Our studies thus far indicate that the severe intestinal aganglionosis of double mutant embryos (*Ret*$^{51/51}$; *Et-3*$^{+/ls}$ and *Ret*$^{51/51}$; *Et-3*$^{ls/ls}$) results from reduced ENCC proliferation. However, an additional explanation could be the requirement of a functional RET/EDNRB interaction to control the migration of ENCCs. Previous studies have shown that RET mediates the chemoattractive and migratory effect of GDNF on ENS progenitors (Natarajan et al., 2002; Young et al., 2001) and that ENCCs from *Ret*^{51} mutants show a diminished response to GDNF (Natarajan et al., 2002). However, the role of EDNRB activation on ENCC migration is presently unknown. To examine this, we analyzed the effect of ET-3 on RET-mediated ENCC chemoattraction on fetal gut explants maintained in collagen gel matrices for 16 hr, as described previously (Natarajan et al., 2002). As expected, addition of GDNF to the culture medium resulted in significant emigration of ENCCs away from the explant and into the collagen matrix (Figure 5; compare panels B and C). However, addition of ET-3 (100 nM) led to a dramatic reduction in the number of ENCCs emigrating from the explants in response to GDNF (Figure 5D). In the absence of GDNF, ET-3 had no discernible effect on ENCC emigration from the explant (not shown). BQ788 had no effect on GDNF-induced ENCC migration (not shown) but abrogated the inhibitory effect of ET-3

in this assay (Figure 5E). These findings indicate that ET-3 inhibits the GDNF-induced migratory response of ENCCs in vitro.

To examine whether the effect of ET-3 on cell migration is mediated via its ability to modulate the activity of PKA, similar explant culture assays were performed in the presence of PKAi or dbcAMP. We found that PKAi abrogates the chemoattractive effect of GDNF on SI explants in a similar manner to ET-3 (Figure 5F). In contrast, db-cAMP appeared to enhance the effect of GDNF (data not shown). These data suggest that, as is the case for cell proliferation, the effect of ET-3 on cell migration is likely to be mediated by changes in PKA activity.

Discussion

Genetic Interaction between *c-Ret* and *Et-3*

The independent roles of the RET and EDNRB signaling pathways in the development of the mammalian ENS and their link to HSCR are well established (Chakravarti, 2001). Over the last several years, a series of murine *c-Ret* alleles have been generated that result in a spectrum of ENS phenotypes, ranging from a small transient reduction in the rate of migration of ENCCs (*Ret*^{9}; de Graaff et al., 2001, and our unpublished observations) to total intestinal aganglionosis (*Ret*$^{k-}$ and *Ret*$^{Tau\text{-}GFP}$; Enomoto et al., 2001; Schuchardt et al., 1994). Between these extremes is the phenotype of *Ret*^{51} homozygous mice, which are characterized by aganglionosis of the colon and constitute a model for HSCR (de Graaff et al., 2001). This phenotype is also observed in mutations of *Ednrb* or *Et-3*, analysis of which has suggested that EDNRB function in ENCCs is required between E11.5 and E12.5 as they cross the cecum and enter the LI (Baynash et al., 1994; Coventry et al., 1994; Hosoda et al., 1994; Kapur et al., 1992; Shin et al., 1999; Sidebotham et al., 2002; Woodward et al., 2000). Together, these studies have suggested a specific requirement of EDNRB activity for enteric ganglia formation in the distal colon (Leibl et al., 1999; Sidebotham et al., 2002; Woodward et al., 2000). Recently, Chakravarti and colleagues have demonstrated cotransmission of *RET* and *EDNRB* alleles in HSCR patients and that a genetic interaction between a null *Ret* and an *Ednrb* loss-of-function allele determines the extent of aganglionosis in the mouse colon (Carrasquillo et al., 2002; McCallion et al., 2003). Here, we have re-examined the expression of *Et-3* and *Ednrb* in mouse embryonic gut during ENCC colonization and analyzed the development of ENS in embryos expressing combinations of *Et-3*^{ls} and *Ret*^{51} alleles. Our results extend previous studies by demonstrating a genetic interaction between *Ret* and *Et-3*, the locus encoding the functional ligand of EDNRB in the gut, and provide evidence that the requirement for ET-3 activity within the fetal gut is not restricted to the colon but extends into prececal gut segments. We suggest that the coordinate activity of RET and EDNRB signaling pathways controls neurogenesis throughout the murine intestine.

The present studies highlight the unique advantages of *Ret*^{51} relative to other *c-Ret* alleles (such as the null *Ret*$^{k-}$) in testing specific interactions with genes encoding components of the ET-3/EDNRB signaling pathway. We reason that the complete absence of ENCCs in *Ret*$^{k-}$ homozygous mice precludes the analysis of the effects on ENS development of this genotype when combined with other unlinked mutations. On the other hand, the lack of a detectable ENS phenotype in *Ret*$^{k-}$ heterozygotes (Durbec et al., 1996; Schuchardt et al., 1994; and our unpublished observations) is consistent with the relatively small effect of a single copy of *Ret*$^{k-}$ on the phenotype of *Ednrb*^{s} or *Ednrb*$^{s\text{-}l}$ mutations (Carrasquillo et al., 2002; McCallion et al., 2003). We suggest that the activity of the hypomorphic *Ret*^{51} allele is sufficiently high to allow formation of enteric ganglia in most of the GI tract of *Ret*$^{51/51}$ animals (de Graaff et al., 2001; and this study) but low enough to provide a sensitive measure of the extent of interaction between *Ret* and *Et-3*. In that respect, it would be interesting to examine the potential interaction of *Ret*^{51} with mutant alleles encoding components of additional independent signaling pathways.

RET and EDNRB Cooperatively Control the Proliferation of ENS Progenitors

Based on the ability of ET-3 to inhibit neuronal differentiation in vitro, it has been suggested that the aganglionosis in *Et-3*$^{ls/ls}$ mutants is due to premature exit from the cell cycle and enhanced differentiation of ENCCs which, in the absence of ET-3, fail to generate enough progenitors to colonize the entire colon (Hearn et al., 1998; Wu et al., 1999). Our studies suggest that, in addition to its antagonistic effect on differentiation, ET-3 cooperates with GDNF to promote the proliferation of undifferentiated ENS progenitors. This early response of ENCCs (observed within 16 hr of culture) does not appear to be the consequence of inhibition of cell differentiation (which reaches maximum effect over a period of 2–7 days) (Hearn et al., 1998; Wu et al., 1999; our unpublished data). Also, the enhancing effect of ET-3 on cell proliferation is unlikely to reflect an increase in cell survival, as deletion of the EDNRB signaling pathway in vivo does not alter apoptotic cell death of neural crest derivatives (Lee et al., 2003). Therefore, the early colonization defect of the gut in *Et-3*$^{ls/ls}$ embryos (Figures 2G, 2H1, and 2H2) and the absence of enteric ganglia in mature animals are likely to result from the combined effects of reduced proliferation and increased differentiation of ENCCs. Our findings differ from previous reports, which showed no clear effect of ET-3 on the proliferation of cultured ENS precursor cells (Hearn et al., 1998; Wu et al., 1999). However, as these studies did not distinguish between uncommitted ENS progenitors and enteric neuroblasts/neurons, it is likely that the presence of the latter cell population (which appear to be refractory to ET-3) obscured the effect of this molecule on the relatively small subpopulation of uncommitted progenitors.

The enhancing effect of ET-3 on the mitotic activity of ENS progenitors is conditional upon activation of RET, highlighting the essential function of this receptor in the proliferation of ENCCs. Consistent with our data, a recent report has demonstrated that GDNF determines the number of enteric neurons in the mouse gut by controlling the proliferation of their progenitors (Gianino et al., 2003). This, together with our studies using wild-type and mutant embryos, suggest strongly that during

enteric neurogenesis, the RET- and EDNRB-mediated signaling pathways have partially overlapping functions and control cooperatively the proliferation of ENS progenitors in vivo.

The critical role of RET on the proliferation of ENS progenitors provides a potential explanation for the transient nature of the colonization defect observed in the SI of E10.5–E12.5 *Et-3*$^{ls/ls}$ mutants. We suggest that in the absence of ET-3, normal RET activation is sufficient to compensate for the lack of EDNRB signaling in the SI. However, removal of ET-3 activity in *Ret*$^{51/51}$ embryos, the cells of which show reduced response to GDNF (Natarajan et al., 2002), is likely to be associated with further critical reduction of early proliferative signals, leading to total intestinal aganglionosis. But why does normal RET signaling fail to rescue the aganglionosis in the colon of ET-3-deficient embryos? It is possible that the relatively high level of GDNF at the cecum and proximal colon during embryogenesis (Leibl et al., 1999; Natarajan et al., 2002; Young et al., 2001) promotes further differentiation of the migrating ENCCs, thus removing an even larger number of ENS progenitors from the cell cycle. Further support for this hypothesis is provided by the analysis of *Ret*$^{+/51}$; *Et-3*$^{ls/ls}$ embryos, which generally show reduced aganglionosis relative to *Et-3*$^{ls/ls}$ mutants. We propose that the partial reduction in RET activity in these embryos compromises the differentiation-promoting effect of RET in ENCCs as they cross the cecum and proximal colon. Although in an otherwise normal genetic background (*Ret*$^{+/51}$; *Et-3*$^{+/+}$) this would have no obvious effect, it is likely that in ET-3-deficient embryos (*Ret*$^{+/51}$; *Et-3*$^{ls/ls}$) it allows a larger fraction of ENS progenitors to remain undifferentiated and retain their migratory capacity. Taken together, our analysis of double mutant embryos indicates that normal colonization of the mammalian intestine by ENCCs and enteric neurogenesis requires the coordinate and balanced interaction between RET and EDNRB signaling pathways. Furthermore, these studies reveal previously unrecognized complexities in the regulation and integration of cellular processes underlying the colonization of the gut by ENCCs and mammalian ENS development.

A large number of in vitro and in vivo studies have identified growth factor requirements for the differentiation of neural crest cells to specific cell lineages (Morrison et al., 2000, and references therein). However, the signals that control proliferation and self-renewal of undifferentiated neural crest cells are less well defined. Such signals are likely to play a critical role in vertebrate development by controlling the number of progenitor cells available for differentiation into the various neural crest derivatives, such as cranial bone and cartilage, melanocytes, and neurons and glia of the peripheral nervous system (Le Douarin and Kalcheim, 1999). Our present data showing that ET-3, in conjunction with GDNF, specifically promotes the proliferation of uncommitted ENS progenitors, together with previous studies demonstrating an inhibitory effect on neuronal differentiation, suggest that endothelins represent a class of extracellular signals that regulate the number of undifferentiated neuroectodermal progenitors. In support of this hypothesis, cell culture studies have previously shown that ET-3 promotes the proliferation of avian primary neural crest cells and melanoblasts (Dupin et al., 2001; Hou et al., 2000; Lahav et al., 1996, 1998; Reid et al., 1996). Furthermore, analysis of mandibular bone phenotypes of zebrafish embryos lacking endothelin-1 suggests that this cytokine regulates the number of embryonic cells recruited as osteoblasts for the formation of specific cranial bones (Kimmel et al., 2003). Therefore, it is likely that endothelins function throughout vertebrate evolution to control the number of progenitors and thus the ultimate size of specific neuroectodermal derivatives, including the ENS. Interestingly, Reid et al. have shown that ET-3, in controlling the proliferation of melanocyte progenitors, acts synergistically with steel factor, the ligand of the RTK KIT (Reid et al., 1996). This, together with our present data, argues that different neural crest lineages have adopted similar molecular strategies for the regulation of progenitor cell numbers.

Activation of EDNRB Inhibits the Chemoattractive Effect of GDNF on ENS Progenitors

Our experiments reveal a previously unrecognized function of ET-3, namely inhibition of GDNF-induced cell migration. The antagonistic effect of ET-3 on the migratory response of ENCCs to RET activation appears to be paradoxical in that *Et-3* is expressed at highest levels near and ahead of the front of migration of ENCCs. However, it is possible that expression of *Et-3* by the gut mesenchyme is critical for setting up the pace and boundaries of ENCC migration. Such restrictions might be important for the orderly colonization of the gut by neural crest cells, and their absence could lead to aberrant migration and abnormal location of ENCCs and their derivatives. In that respect, it is interesting that in ET-3-deficient mice, ectopic ganglia have been identified in the pelvic region located outside the enteric musculature in the adventitia of the colon (Payette et al., 1987; Rothman and Gershon, 1984). Such ganglia could be derived from ENS progenitors that fail to follow the correct migratory pathways established by GDNF.

PKA Is Likely to Mediate Some of the Effects of ET-3 on RET Signaling

Our experiments suggest that PKA is likely to be an important component of the regulatory link between the signaling pathways activated by RET and EDNRB. Specific inhibition of PKA activity mimics the effect of ET-3 on GDNF-induced cell proliferation and migration, while activation of PKA by pharmacological agents that increase intracellular levels of cAMP is sufficient to block these effects. Moreover, increased intracellular levels of cAMP prevent the proliferative effect of ET-3, suggesting that ET-3 normally functions by reducing the activity of PKA in EDNRB-expressing cells. Further support for such a role comes from the identification of HSCR patients with a mutant form of *EDNRB* that is unable to reduce intracellular cAMP but retains other signaling properties (Fuchs et al., 2001). Recent findings by Takahashi and colleagues suggest a potential mechanism by which modulation of PKA activity could influence aspects of RET function (Fukuda et al., 2002). These workers identified serine 696 (S696) in the juxtamembrane domain of RET as a target of PKA and showed that its phosphorylation promotes lamellipodia formation in neuroectodermal cells by enhancing the

activity of effector signaling pathways downstream of RET (Fukuda et al., 2002). One such signaling pathway is that of PI3K, activation of which is critical for ENCC migration (Natarajan et al., 2002). We therefore suggest that the inhibitory effect of ET-3 on GDNF-induced chemoattraction results from inhibition of PKA and thus the suboptimal activation of effectors of PI3K and possibly other signaling pathways. However, it is currently unclear whether the effects of PKA as a modulator of GDNF-induced ENCC proliferation are mediated by S696 or other potential PKA target sites on RET or other components of its signaling pathway.

Concluding Remarks

Neurogenesis along the gut is an asynchronous process. Due to the single entry point of vagal neural crest cells (anterior foregut) and the extensive length of the gut, the rostrocaudal colonization of the organ by neural crest cells is gradual and completed over several days. During this period, extensive neurogenesis and gangliogenesis is taking place in proximal gut segments while more distal regions are being invaded by the first ENCCs. This mode of development requires that the various cellular processes underlying enteric neurogenesis are coordinated along the gut and integrated with the growth of the organ itself. As RET signaling controls the survival, proliferation, differentiation, and migration of mammalian ENS progenitors, regulation of the outcome of RET activation within the GI tract is likely to be critical for normal ENS development. Such regulation could be achieved by the spatial and temporal control of the expression and activity of other signaling factors along the length of the gut, which could influence the outcome of RET signaling. The dynamic distribution of ET-3 along the gut and its differential effects on cellular processes controlled by RET suggest that this signaling molecule fulfils the criteria as a regulator of RET signaling. Understanding the molecular mechanisms that mediate such control will provide new insights into the process of vertebrate neurogenesis and contribute to the understanding of the pathogenesis of congenital neuronal deficiencies.

Experimental Procedures

Animals

The generation and genotyping of Ret^{51} and Ret^{k-} mice has been described previously (de Graaff et al., 2001; Schuchardt et al., 1994). Mice carrying the $Et\text{-}3^{ls}$ allele were obtained from the Medical Research Council Mammalian Genetics Unit, Harwell (UK) and backcrossed to C57Bl/6 mice for at least four generations. Heterozygous and homozygous $Et\text{-}3^{ls}$ animals were identified by PCR (Rice et al., 2000). Tissues from wild-type animals were obtained either from littermates of mutants (Figure 2) or from Parkes (outbred) mice. The day of vaginal plug was considered to be E0.5.

Explant Cultures

Embryonic gut explant cultures have been described previously (Natarajan et al., 2002). Briefly, gut was dissected from E10.5 mouse embryos, placed in a three-dimensional collagen gel matrix, and cultured overnight in a defined medium (optiMEM: Life Technologies, UK) supplemented with L-glutamine (1 mM; Life Technologies) and penicillin/streptomycin antibiotic mixture (Life Technologies) in an atmosphere of 5% CO_2. Combinations of GDNF (10 ng/ml; Peprotech), ET-3 (100 nM; Calbiochem), PKA inhibitor (4-Cyano-3-methylisoquinoline 100 nM; Calbiochem), forskolin (20 μM; ICN), and db-cAMP (1 mM; Calbiochem) were added to this medium as described.

Cultures of Dissociated Cecum and Small Intestine

The cecal region was dissected from E11.0–E11.5 mouse embryos (Parkes) and incubated with Dispase/Collagenase (0.5 mg/ml in Phosphate Buffered Saline-1× PBS; Roche) for 5 min at room temperature. The tissue was washed three times with 1× PBS, dissociated into single cells by repeated pipetting, and plated onto fibronectin-coated (20 μg/ml) SONIC-SEAL SLIDE wells (VWR) (equivalent of one quarter of a cecum/well) in optiMEM supplemented with combinations of GDNF (10 ng/ml), ET-3 (100 nM), PKAi (100 nM), forskolin (20 μM; ICN), or the PKC inhibitor (bisindolylmaleimide 20 μM; Calbiochem). Cultures were incubated for up to 36 hr in an atmosphere of 5% CO_2.

Acute cultures of dissociated SI were prepared from E11.5–E12.0 wild-type and $Et\text{-}3^{ls/ls}$ mutants as described above. These cultures were maintained in optiMEM for up to 2 hr in an atmosphere of 5% CO_2.

Immunostaining

For immunostaining, explants were fixed for 2 hr in 4% paraformaldehyde (in 1× PBS) at 4°C. After washing twice in PBT (1× PBS+0.1% Triton X-100), they were incubated for 5 hr with a polyclonal anti-RET antibody (goat; R&D Systems, 1:50) and TuJ1 (mouse; BABCO, 1:1000), diluted in PBT containing 1% BSA and 0.15% Glycine (PBG). The explants were then washed three times with PBG and incubated for 2 hr at room temperature with anti-goat FITC-conjugated antiserum (1:500; Jackson Labs) and anti-mouse Alexa Fluor (1:500; Molecular Probes). After washing three times in PBG, explants were mounted in VECTASHIELD (Vector Laboratories) and analyzed with a Zeiss Axiophot compound microscope.

Dissociated cecum cultures were fixed with 3% paraformaldehyde for 15 minutes at 4°C. The cells were subsequently washed three times in PBT and then immunostained with TuJ1 (mouse; BABCO, 1:1000), RET (rabbit; IBL, 1:50), and anti-phospho-Histone-3 (rabbit; Upstate, 1:500) for 5 hr at room temperature in PBT containing 10% heat-inactivated sheep serum (HISS). After washing three times in PBT, the cells were incubated with secondary antibodies (anti-mouse FITC-conjugated; Jackson Labs, 1:500, and anti-rabbit Alexa Fluor; Molecular Probes, 1:500) in 10% HISS/PBT for 2 hr at room temperature. The cells were washed three times in PBT, counterstained with TO-PRO-3-iodide (Molecular Probes, 1:3000 in PBS), and mounted using VECTASHIELD. The cells were analyzed with a BIO-RAD confocal microscope, and images were compiled using Adobe Photoshop software.

Acute cultures of dissociated small intestine were fixed for 10 min in 4% paraformaldehyde at 4°C. After washing three times with PBT, the cells were immunostained with a monoclonal antibody for SOX10 (Kim et al., 2003; mouse; 1:10 kind gift from Dr. D. Anderson) and anti-phospho-Histone-3 (rabbit; Upstate, 1:500) overnight at 4°C. Secondary antibodies (anti-rabbit FITC-conjugated; Jackson Labs, 1:500, and anti-mouse Alexa Fluor; Molecular Probes, 1:500) were applied for 2 hr at room temperature.

In Situ Hybridization

Whole-mount in situ hybridization histochemistry was performed as previously described (Durbec et al., 1996; Natarajan et al., 2002). The riboprobes for detecting *c-Ret* mRNA was generated from pmcRet7 (Durbec et al., 1996; Pachnis et al., 1993). *Phox2b* cRNA was generated from a 1.6 kb cDNA clone (kindly provided by C. Goridis) containing the entire open reading frame and the 3′ untranslated region (Pattyn et al., 1997). Riboprobes for detecting *Et-3* and *EdnrB* were transcribed from cDNA clones containing PCR amplified fragments (kind gifts from M. Gershon). The *Et-3*-specific riboprobe is transcribed from the PCR fragment encompassing nucleotides 372–989 of the full-length cDNA. A PCR fragment containing nucleotides 340–923 was used to prepare the *EdnrB*-specific riboprobe. For sectioning, whole-mount preparations were postfixed in 4% paraformaldehyde for 1 hr, cryoprotected with 30% sucrose in 1× PBS, embedded in OCT, and sectioned at 12 μ.

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References

Airaksinen, M.S., Titievsky, A., and Saarma, M. (1999). GDNF family neurotrophic factor signaling: four masters, one servant? Mol. Cell. Neurosci. *13*, 313–325.

Baloh, R.H., Enomoto, H., Johnson, E.M., Jr., and Milbrandt, J. (2000). The GDNF family ligands and receptors—implications for neural development. Curr. Opin. Neurobiol. *10*, 103–110.

Baynash, A.G., Hosoda, K., Giaid, A., Richardson, J.A., Emoto, N., Hammer, R.E., and Yanagisawa, M. (1994). Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. Cell *79*, 1277–1285.

Cacalano, G., Farinas, I., Wang, L.C., Hagler, K., Forgie, A., Moore, M., Armanini, M., Phillips, H., Ryan, A.M., Reichardt, L.F., et al. (1998). GFRalpha1 is an essential receptor component for GDNF in the developing nervous system and kidney. Neuron *21*, 53–62.

Carrasquillo, M.M., McCallion, A.S., Puffenberger, E.G., Kashuk, C.S., Nouri, N., and Chakravarti, A. (2002). Genome-wide association study and mouse model identify interaction between RET and EDNRB pathways in Hirschsprung disease. Nat. Genet. *32*, 237–244.

Chakravarti, A.L. (2001). Hirschsprung's disease. In The Metabolic and Molecular Bases of Inherited Diseases, C.R. Scriver, A.L. Beaudet, D. Valle, W.S. Sly, B. Childs, K. Kinzler, and B. Vogelstein, eds. (New York: McGraw-Hill), pp. 6231–6255.

Coventry, S., Yost, C., Palmiter, R.D., and Kapur, R.P. (1994). Migration of ganglion cell precursors in the ileoceca of normal and lethal spotted embryos, a murine model for Hirschsprung disease. Lab. Invest. *71*, 82–93.

de Graaff, E., Srinivas, S., Kilkenny, C., D'Agati, V., Mankoo, B.S., Costantini, F., and Pachnis, V. (2001). Differential activities of the RET tyrosine kinase receptor isoforms during mammalian embryogenesis. Genes Dev. *15*, 2433–2444.

Dupin, E., Real, C., and Ledouarin, N. (2001). The neural crest stem cells: control of neural crest cell fate and plasticity by endothelin-3. An. Acad. Bras. Cienc. *73*, 533–545.

Durbec, P.L., Larsson-Blomberg, L.B., Schuchardt, A., Costantini, F., and Pachnis, V. (1996). Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. Development *122*, 349–358.

Enomoto, H., Araki, T., Jackman, A., Heuckeroth, R.O., Snider, W.D., Johnson, E.M., Jr., and Milbrandt, J. (1998). GFR alpha1-deficient mice have deficits in the enteric nervous system and kidneys. Neuron *21*, 317–324.

Enomoto, H., Crawford, P.A., Gorodinsky, A., Heuckeroth, R.O., Johnson, E.M., Jr., and Milbrandt, J. (2001). RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development *128*, 3963–3974.

Focke, P.J., Swetlik, A.R., Schilz, J.L., and Epstein, M.L. (2003). GDNF and insulin cooperate to enhance the proliferation and differentiation of enteric crest-derived cells. J. Neurobiol. *55*, 151–164.

Fuchs, S., Amiel, J., Claudel, S., Lyonnet, S., Corvol, P., and Pinet, F. (2001). Functional characterization of three mutations of the endothelin B receptor gene in patients with Hirschsprung's disease: evidence for selective loss of Gi coupling. Mol. Med. *7*, 115–124.

Fukuda, T., Kiuchi, K., and Takahashi, M. (2002). Novel mechanism of regulation of Rac activity and lamellipodia formation by RET tyrosine kinase. J. Biol. Chem. *277*, 19114–19121.

Gershon, M.D. (1999). Endothelin and the development of the enteric nervous system. Clin. Exp. Pharmacol. Physiol. *26*, 985–988.

Gershon, M.D., Kirchgessner, A.L., and Wade, P.R. (1994). Functional anatomy of the enteric nervous system. In Physiology of the Gastrointestinal Tract, L.R. Johnson, ed. (New York: Raven Press), pp. 381–422.

Gianino, S., Grider, J.R., Cresswell, J., Enomoto, H., and Heuckeroth, R.O. (2003). GDNF availability determines enteric neuron number by controlling precursor proliferation. Development *130*, 2187–2198.

Hearn, C.J., Murphy, M., and Newgreen, D. (1998). GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro. Dev. Biol. *197*, 93–105.

Heuckeroth, R.O., Lampe, P.A., Johnson, E.M., and Milbrandt, J. (1998). Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. Dev. Biol. *200*, 116–129.

Hosoda, K., Hammer, R.E., Richardson, J.A., Baynash, A.G., Cheung, J.C., Giaid, A., and Yanagisawa, M. (1994). Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. Cell *79*, 1267–1276.

Hou, L., Panthier, J.J., and Arnheiter, H. (2000). Signaling and transcriptional regulation in the neural crest-derived melanocyte lineage: interactions between KIT and MITF. Development *127*, 5379–5389.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., and Masaki, T. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc. Natl. Acad. Sci. USA *86*, 2863–2867.

James, A.F., Xie, L.H., Fujitani, Y., Hayashi, S., and Horie, M. (1994). Inhibition of the cardiac protein kinase A-dependent chloride conductance by endothelin-1. Nature *370*, 297–300.

Jiao, X., Gonzalez-Cabrera, P.J., Xiao, L., Bradley, M.E., Abel, P.W., and Jeffries, W.B. (2002). Tonic inhibitory role for cAMP in alpha(1a)-adrenergic receptor coupling to extracellular signal-regulated kinases 1/2. J. Pharmacol. Exp. Ther. *303*, 247–256.

Kapur, R.P., Yost, C., and Palmiter, R.D. (1992). A transgenic model for studying development of the enteric nervous system in normal and aganglionic mice. Development *116*, 167–175.

Kim, J., Lo, L., Dormand, E., and Anderson, D.J. (2003). SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. Neuron *38*, 17–31.

Kimmel, C.B., Ullmann, B., Walker, M., Miller, C.T., and Crump, J.G. (2003). Endothelin 1-mediated regulation of pharyngeal bone development in zebrafish. Development *130*, 1339–1351.

Lahav, R., Ziller, C., Dupin, E., and Le Douarin, N.M. (1996). Endothelin 3 promotes neural crest cell proliferation and mediates a vast increase in melanocyte number in culture. Proc. Natl. Acad. Sci. USA *93*, 3892–3897.

Lahav, R., Dupin, E., Lecoin, L., Glavieux, C., Champeval, D., Ziller, C., and Le Douarin, N.M. (1998). Endothelin 3 selectively promotes survival and proliferation of neural crest-derived glial and melanocytic precursors in vitro. Proc. Natl. Acad. Sci. USA *95*, 14214–14219.

Le Douarin, N., and Kalcheim, C. (1999). The Neural Crest, 2nd edn (Cambridge, UK: Cambridge University Press).

Lee, H.O., Levorse, J.M., and Shin, M.K. (2003). The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors. Dev. Biol. *259*, 162–175.

Leibl, M.A., Ota, T., Woodward, M.N., Kenny, S.E., Lloyd, D.A., Vaillant, C.R., and Edgar, D.H. (1999). Expression of endothelin 3 by mesenchymal cells of embryonic mouse caecum. Gut *44*, 246–252.

Lyon, M., Rastan, S., and Brown, S.D.M. ed. (1996). Genetic Variants and Strains of the Laboratory Mouse (Oxford, UK: Oxford University Press).

McCallion, A.S., Stames, E., Conlon, R.A., and Chakravarti, A. (2003). Phenotype variation in two-locus mouse models of Hirschsprung disease: tissue-specific interaction between Ret and Ednrb. Proc. Natl. Acad. Sci. USA *100*, 1826–1831.

Moore, M.W., Klein, R.D., Farinas, I., Sauer, H., Armanini, M., Phillips,

H., Reichardt, L.F., Ryan, A.M., Carver-Moore, K., and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. Nature *382*, 76–79.

Morrison, S.J., Perez, S.E., Qiao, Z., Verdi, J.M., Hicks, C., Weinmaster, G., and Anderson, D.J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. Cell *101*, 499–510.

Natarajan, D., Marcos-Gutierrez, C., Pachnis, V., and De Graaff, E. (2002). Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development *129*, 5151–5160.

Ono, K., Tsujimoto, G., Sakamoto, A., Eto, K., Masaki, T., Ozaki, Y., and Satake, M. (1994). Endothelin-A receptor mediates cardiac inhibition by regulating calcium and potassium currents. Nature *370*, 301–304.

Pachnis, V., Mankoo, B., and Costantini, F. (1993). Expression of the c-ret proto-oncogene during mouse embryogenesis. Development *119*, 1005–1017.

Paratore, C., Goerich, D.E., Suter, U., Wegner, M., and Sommer, L. (2001). Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. Development *128*, 3949–3961.

Paratore, C., Eichenberger, C., Suter, U., and Sommer, L. (2002). Sox10 haploinsufficiency affects maintenance of progenitor cells in a mouse model of Hirschsprung disease. Hum. Mol. Genet. *11*, 3075–3085.

Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J.F. (1997). Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. Development *124*, 4065–4075.

Payette, R.F., Tennyson, V.M., Pham, T.D., Mawe, G.M., Pomeranz, H.D., Rothman, T.P., and Gershon, M.D. (1987). Origin and morphology of nerve fibers in the aganglionic colon of the lethal spotted (ls/ls) mutant mouse. J. Comp. Neurol. *257*, 237–252.

Pichel, J.G., Shen, L., Sheng, H.Z., Granholm, A.C., Drago, J., Grinberg, A., Lee, E.J., Huang, S.P., Saarma, M., Hoffer, B.J., et al. (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. Nature *382*, 73–76.

Reid, K., Turnley, A.M., Maxwell, G.D., Kurihara, Y., Kurihara, H., Bartlett, P.F., and Murphy, M. (1996). Multiple roles for endothelin in melanocyte development: regulation of progenitor number and stimulation of differentiation. Development *122*, 3911–3919.

Rice, J., Doggett, B., Sweetser, D.A., Yanagisawa, H., Yanagisawa, M., and Kapur, R.P. (2000). Transgenic rescue of aganglionosis and piebaldism in lethal spotted mice. Dev. Dyn. *217*, 120–132.

Rothman, T.P., and Gershon, M.D. (1984). Regionally defective colonization of the terminal bowel by the precursors of enteric neurons in lethal spotted mutant mice. Neuroscience *12*, 1293–1311.

Saarma, M. (2000). GDNF—a stranger in the TGF-beta superfamily? Eur. J. Biochem. *267*, 6968–6971.

Sanchez, M.P., Silos-Santiago, I., Frisen, J., He, B., Lira, S.A., and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature *382*, 70–73.

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F., and Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature *367*, 380–383.

Shin, M.K., Levorse, J.M., Ingram, R.S., and Tilghman, S.M. (1999). The temporal requirement for endothelin receptor-B signalling during neural crest development. Nature *402*, 496–501.

Sidebotham, E.L., Woodward, M.N., Kenny, S.E., Lloyd, D.A., Vaillant, C.R., and Edgar, D.H. (2002). Localization and endothelin-3 dependence of stem cells of the enteric nervous system in the embryonic colon. J. Pediatr. Surg. *37*, 145–150.

Taraviras, S., and Pachnis, V. (1999). Development of the mammalian enteric nervous system. Curr. Opin. Genet. Dev. *9*, 321–327.

Taraviras, S., Marcos-Gutierrez, C.V., Durbec, P., Jani, H., Grigoriou, M., Sukumaran, M., Wang, L.C., Hynes, M., Raisman, G., and Pachnis, V. (1999). Signalling by the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system. Development *126*, 2785–2797.

Tokuda, H., Harada, A., Hirade, K., Matsuno, H., Ito, H., Kato, K., Oiso, Y., and Kozawa, O. (2003). Incadronate amplifies prostaglandin F2alfa-induced vascular endothelial growth factor synthesis in osteoblasts: enhancement of MAP kinase activity. J. Biol. Chem. *278*, 18930–18937.

Woodward, M.N., Kenny, S.E., Vaillant, C., Lloyd, D.A., and Edgar, D.H. (2000). Time-dependent effects of endothelin-3 on enteric nervous system development in an organ culture model of Hirschsprung's disease. J. Pediatr. Surg. *35*, 25–29.

Worley, D.S., Pisano, J.M., Choi, E.D., Walus, L., Hession, C.A., Cate, R.L., Sanicola, M., and Birren, S.J. (2000). Developmental regulation of GDNF response and receptor expression in the enteric nervous system. Development *127*, 4383–4393.

Wu, J.J., Chen, J.X., Rothman, T.P., and Gershon, M.D. (1999). Inhibition of in vitro enteric neuronal development by endothelin-3: mediation by endothelin B receptors. Development *126*, 1161–1173.

Young, H.M., Hearn, C.J., Ciampoli, D., Southwell, B.R., Brunet, J., and Newgreen, D.F. (1998). A single rostrocaudal colonization of the rodent intestine by enteric neuron precursors is revealed by the expression of phox2b, ret, and p75 and by explants grown under the kidney capsule or in organ culture. Dev. Biol. *202*, 67–84.

Young, H.M., Ciampoli, D., Hsuan, J., and Canty, A.J. (1999). Expression of Ret-, p75(NTR)-, Phox2a-, Phox2b-, and tyrosine hydroxylase-immunoreactivity by undifferentiated neural crest-derived cells and different classes of enteric neurons in the embryonic mouse gut. Dev. Dyn. *216*, 137–152.

Young, H.M., Hearn, C.J., Farlie, P.G., Canty, A.J., Thomas, P.Q., and Newgreen, D.F. (2001). GDNF is a chemoattractant for enteric neural cells. Dev. Biol. *229*, 503–516.

Young, H.M., Bergner, A.J., and Muller, T. (2003). Acquisition of neuronal and glial markers by neural crest-derived cells in the mouse intestine. J. Comp. Neurol. *456*, 1–11.