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Sox10 haploinsufficiency affects maintenance of progenitor cells in a mouse model of Hirschsprung disease

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Hirschsprung disease, or congenital megacolon, is characterized by aganglionosis of the terminal bowel, which leads to intestinal obstruction and chronic constipation. Several genes involved in the disease have been identified. In particular, haploinsufficiency of *SOX10*, which encodes a transcription factor, results in megacolon, often in combination with other disorders. Although Hirschsprung disease has been recognized as a neurocristopathy, the cellular mechanisms that lead to aganglionosis in affected individuals are unclear. Failure of mutant enteric progenitor cells to migrate into the gut, to survive, or to differentiate into appropriate cell types at the appropriate time and in correct numbers might contribute to the disease phenotype. In the present study, we use mice with a targeted deletion of *Sox10* to study the etiology of Hirschsprung disease. We demonstrate that neural crest-derived enteric progenitors that are heterozygous for the *Sox10* mutation colonize the proximal intestine and are unaffected in their survival capacity. However, unlike their wild-type counterparts, mutant enteric neural crest-derived cells are unable to maintain their progenitor state and acquire preneuronal traits, which results in a reduction of the progenitor pool size. Thus, the cells that normally colonize the hindgut are depleted in the *Sox10* mutant, causing the distal bowel to become aganglionic.

INTRODUCTION

Hirschsprung disease is a relatively common congenital disorder that is defined by the absence of enteric ganglia in the myenteric and submucosal plexuses along a variable length of the distal gut (reviewed in 1). The abnormal innervation and absence of peristaltic movement cause severe constipation, which results in dilated megacolon. So far, the disorder can only be treated by surgical resection of the affected bowel. The disease has a prevalence of about 1 in 5000 infants with variable penetrance that is, however, greater in males than in females. The inheritance pattern is mostly complex, indicating the multifactorial nature of the disease. A recent genetic analysis of Hirschsprung disease has revealed that a form of this disorder can be explained by the interaction of three loci, one of which is *RET* (2). Mutations in *RET*, which encodes a receptor tyrosine kinase, are responsible for most cases of Hirschsprung disease (reviewed in 3,4). Moreover, a few patients have been identified with mutations in genes encoding glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN), both of which signal through RET (5–7). Deficiencies in signaling by endothelin-3 (ET-3) and its receptor endothelin-B (ET_B) have also been implicated in congenital megacolon, both in mice and in human patients (reviewed in 8). In addition to defects in ligand–receptor pairs, mutations in two transcription factors have been shown to result in aganglionosis of the terminal bowel in human patients. *SIP1* encodes a Smad interacting protein that when mutated is responsible for a form of Hirschsprung disease associated with mental retardation and microcephaly (9,10). Finally, haploinsufficiency of *SOX10*, encoding a transcriptional modulator with a Sry-type HMG domain, leads to Hirschsprung disease (11,12). Frequently, affected patients also display pigment anomalies and deafness, traits that are, in combination with enteric aganglionosis, characteristic of the so-called Waardenburg syndrome type 4 (WS4). Some patients with *SOX10* mutations moreover suffer from myelin deficiencies beside the classical symptoms of WS4 (13–16).

The availability of mouse models has greatly facilitated the analysis of the mechanisms causing Hirschsprung disease. Both spontaneous mouse mutations and targeted deletion have confirmed the importance of Hirschsprung disease genes in the formation of the enteric nervous system (ENS) and helped to identify Hirschsprung disease as a neurocristopathy (reviewed in 1). In particular, dominant megacolon (DOM), a spontaneous mutation of *Sox10* (17,18), and a null mutation of *Sox10* generated by targeted deletion (19) lead to a phenotype very similar to WS4 observed in human patients carrying *SOX10* mutations. Moreover, as in human patients, enteric

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aganglionosis and failures in pigmentation are caused by *Sox10* haploinsufficiency and show variable penetrance, depending on the genetic background (17–21). These features make *Sox10* mouse mutants a valuable model system to study the etiology of Hirschsprung disease. Homozygosity for a *Sox10* mutation leads to the absence of most neural crest derivatives (17–19,22). This phenotype, together with the finding that Sox10 is expressed in virtually all neural crest stem cells both during migration and in postmigratory neural crest target structures (23,24), indicated that Sox10 plays a role at earliest stages of neural crest development. In agreement with this hypothesis, analysis of the developmental potential of *Sox10*-mutant neural crest cells revealed a dual requirement for Sox10 in regulating survival and fate decision processes (24).

Most of the ENS is generated by multipotent progenitor cells derived from the vagal neural crest that enter the mesenchyme of the foregut and then migrate in a proximal–distal direction to eventually colonize the hindgut (1,25). Moreover, a minor portion of the ENS is formed by cells derived from the sacral neural crest. Thus, given the above-mentioned role of Sox10 in neural crest cells, it is possible that cell death, aberrant fate decisions and premature differentiation of neural crest cells colonizing the intestine might lead to enteric aganglionosis in individuals suffering from Hirschsprung disease. Alternatively, deficient migration of enteric progenitor cells might prevent their localization to the mutant hindgut, or progenitors able to reach the distal gut might fail to undergo differentiation (8). In the present study, we addressed these issues in mice heterozygous for a targeted deletion of *Sox10* (19).

RESULTS

Aganglionosis in the hindgut of *Sox10* heterozygous mutant animals

The phenotypes of $Sox10^{Dom}$ mice appear to be identical to the phenotypes observed in mice carrying a targeted *Sox10* mutation ($Sox10^{lacZ}$ mice) (19). In particular, similar to heterozygous $Sox10^{Dom}$ mice (20,26,27), heterozygous $Sox10^{lacZ}$ mice develop megacolon which causes death during the first postnatal weeks in a significant fraction of the mutant animals (19). In $Sox10^{Dom}$ mice, the development of megacolon is a consequence of aganglionosis or hypoganglionosis that is already established at embryonic stages (20,26). The penetrance of this phenotype is modulated by the genetic background but is highest in first-generation offspring from breedings of $Sox10^{Dom/+}$ males (on a mixed C57Bl/6J and C3HeB/FeJ background) with C57Bl/6J females (20). To investigate the enteric nervous system (ENS) of heterozygous $Sox10^{lacZ}$ mutant embryos, we mated $Sox10^{lacZ/+}$ males (that had been back-crossed with C3HeB/FeJ mice for several generations (19)) with C57Bl/6J females. *In situ* hybridization experiments revealed that the hindgut of heterozygous progeny of such matings displayed an absence or reduced number of neuronal cells marked by the receptor tyrosine kinase c-Ret (Fig. 1A and B), neurofilament 160 (NF) (Fig. 1C and D) and peripherin (data not shown). In contrast, the number of enteric ganglion cells appeared to be comparable in more proximal parts of wild-type and heterozygous mutant gut (Fig. 1A and B).

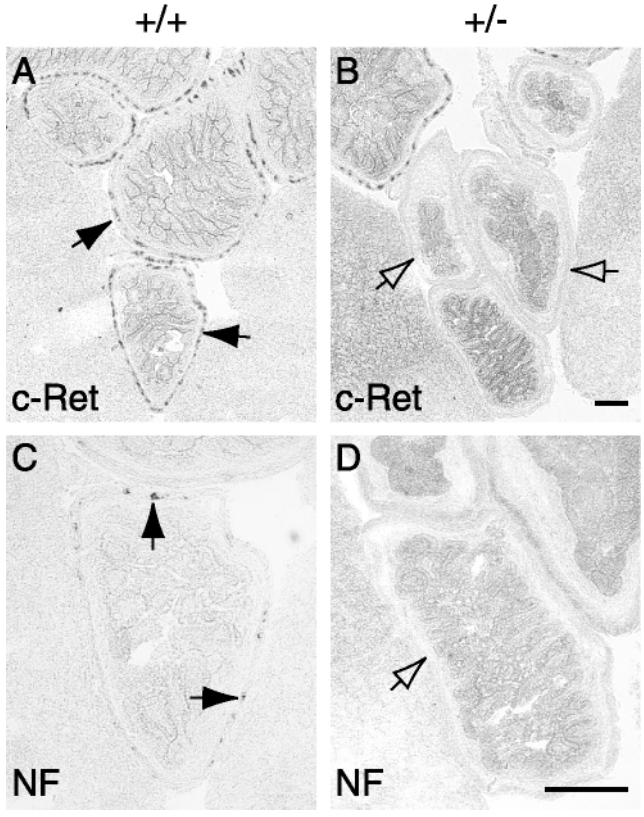

Figure 1. $Sox10^{lacZ/+}$ mice display aganglionosis in the hindgut. *In situ* hybridization analysis on E17 cross-sections revealed that the gut of $Sox10^{+/+}$ animals (+/+) is entirely innervated by neurons as assessed by c-Ret (**A**; arrows) and neurofilament 160 (NF) (**C**; arrows) expression. In contrast, the neuronal markers c-Ret (**B**; open arrows) and NF (**D**; open arrow) are absent in the distal gut of a $Sox10^{lacZ/+}$ animal (+/−), indicating that the mutant animal lacks enteric neurons in the hindgut. More proximal parts of the gut are normally innervated. Scale bars: 100 μm.

Reduction of progenitor cell numbers at early stages of ENS development in *Sox10*-heterozygous mutants

Sox10 is expressed in multipotent neural crest cells but is downregulated as these cells adopt a neuronal fate (23,24). Therefore, the loss of neuronal cells in the ENS of *Sox10*-heterozygous mice is likely to be secondary to a defect in multipotent neural crest-derived enteric progenitors. To address this question, we investigated the expression of progenitor markers in wild-type and mutant gut at early developmental stages. Sox10 itself is a reliable marker for migratory and postmigratory multipotent neural crest cells (24). Semiquantitative RT–PCR analysis has previously indicated that Sox10 mRNA levels are reduced by approximately half in heterozygous as compared with wild-type embryos (19). Nonetheless, Sox10 transcripts are readily detectable by *in situ* hybridization experiments in migratory neural crest cells and in progenitors present in peripheral ganglia and nerves of $Sox10^{lacZ/+}$ embryos (Fig. 2A and B) (17). Similarly, the tyrosine kinase receptor ErbB3 is a marker for multipotent neural crest cells and peripheral glia, which is expressed at comparable levels in wild-type and *Sox10*-heterozygous mutant

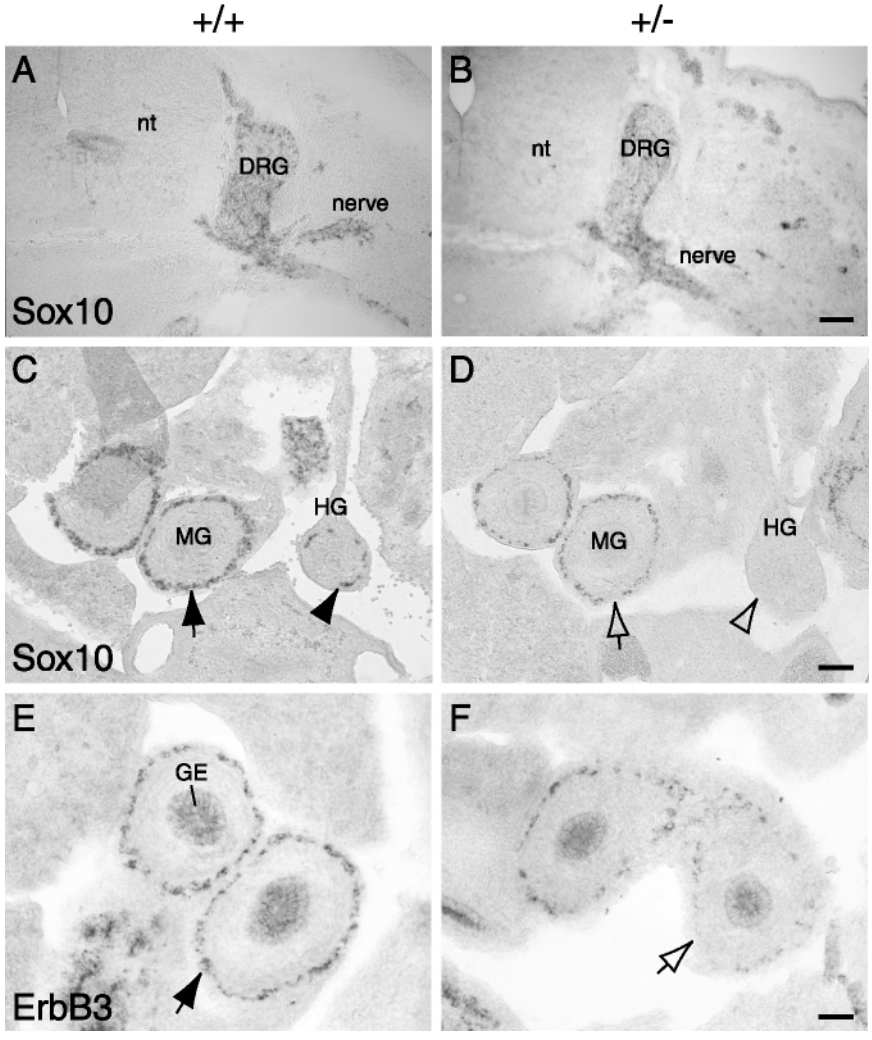

Figure 2. The hindgut of *Sox10*$^{lacZ/+}$ mutants is not colonized by enteric progenitor cells and the number of progenitors in more proximal parts of the gut is reduced. *In situ* hybridization studies showed readily detectable Sox10 expression levels in dorsal root ganglia (DRG) and peripheral nerve of wild-type (+/+) and mutant (+/−) embryos at E13 (**A**, **B**). nt, neural tube. In contrast, the hindgut (HG) of *Sox10*$^{lacZ/+}$ animals at E13 is devoid of Sox10 expression (**D**; open arrowhead). At this developmental stage, the wild-type hindgut is colonized by Sox10-positive cells (**C**; arrowhead). At the level of duodenum and midgut (MG), the expression levels of the progenitor markers Sox10 and ErbB3 are reduced in the mutant (open arrows in D, **F**) as compared with the wild-type (arrows in C, **E**). Note the unaltered expression levels of ErbB3 in the mutant gut epithelium (GE) (compare E and F). Scale bars: (B, D) 200 µm; (F) 100 µm.

neural crest cells (24). In contrast to DRG, peripheral nerves and other neural crest-derived structures, the hindgut was devoid of Sox10- and ErbB3-expressing cells at E13 in five of five *Sox10*-mutant embryos tested (open arrowhead in Fig. 2D; and data not shown), in agreement with a previous study performed with *Sox10*$^{Dom/+}$ mice (27). At this stage, wild-type hindgut has already been colonized by enteric progenitor cells (arrowhead in Fig. 2C). Likewise, c-Ret expression was detectable in the hindgut of wild-type but not of *Sox10*-heterozygous mutant embryos at E13 (data not shown). Thus, the impaired formation of an ENS in the distal gut of *Sox10*-mutant animals correlates with the absence of progenitor markers in the hindgut of mutant animals at early developmental stages.

Strikingly, although they were not completely absent as in the hindgut, progenitor cells were reduced in number in the duodenum and in the midgut of five out of five mutant embryos. While normally the developing myenteric plexus of the proximal gut is outlined by multiple progenitor cells forming a continuous ring of Sox10 expression (arrow in Fig. 2C), Sox10-positive cells appeared sparsely distributed in the early ENS of the mutant midgut (open arrow in Fig. 2D). Likewise, higher magnification of proximal gut sections hybridized with an ErbB3 riboprobe revealed that ErbB3 staining was confined to relatively few ENS progenitor cells in the mutant midgut, while many ErbB3-positive cells were detectable on wild-type sections (arrows in Fig. 2E and F).

The apparent reduction of progenitor cells in the midgut and hindgut of *Sox10*-mutant embryos could have two alternative explanations. Enteric progenitor cells might be generated in normal numbers in the mutant, but these cells might for some reason be unable to properly colonize the gut in a proximal to distal direction. Alternatively, the total number of enteric progenitors in the intestine might be decreased and failure to

populate distal gut regions might primarily be due to a loss of progenitors. To address this issue, we quantified the total number of enteric progenitor cells present in whole intestine of wild-type or mutant embryos at different developmental stages. Apart from Sox10 and ErbB3, migratory and postmigratory neural crest stem cells are marked by the low-affinity neurotrophin receptor p75 (28–30) and multipotent enteric progenitors have previously been isolated by virtue of their p75 expression (31,32). Likewise, in *Sox10*$^{lacZ/+}$ animals, p75 expression marks undifferentiated neural crest cells in neural crest explants, dissociated DRG and gut (Fig. 3A–F) (24). Moreover, all undifferentiated mutant neural crest cells prepared from various neural crest targets, including the gut, can also be characterized by Sox10 protein expression (Fig. 3A–F) (24). Therefore, for a quantification of enteric progenitor cells, we dissociated the intestine of wild-type and mutant embryos at different developmental stages, plated the cells onto culture dishes and, 3 h after plating, determined by immunocytochemistry the fraction of cells that were positive for both p75 and Sox10. To account for possible variability in penetrance of the phenotype, whole intestines from three different embryos per stage were analysed. Counting ~1000 cells per embryo at E13 demonstrated that 4.8% of all cells were p75/Sox10-positive multipotent progenitor cells in the wild-type (Fig. 3G). This number was only 3.2% in the *Sox10* mutant, equivalent to a reduction of 33%. At later stages, the loss of enteric progenitors was even more pronounced. At E15, 8.8% of all cells displayed features of progenitor cells in the wild type. In the mutant at E15, however, only 5.2% of all cells were progenitors, corresponding to a reduction of progenitor numbers of 41% (Fig. 3G). Furthermore, at E17, the number of p75/Sox10-positive cells present in whole intestine of *Sox10*-mutant embryos was decreased by 50% (from 6.9% of all cells in the wild type to 3.5% in the mutant; Fig. 3G). Thus, these data confirm the results of our *in vivo* experiments (Fig. 2) and demonstrate that *Sox10* haploinsufficieny leads to a reduced size of the multipotent progenitor pool in the gut.

Sox10 haploinsufficiency does not affect survival of enteric progenitors

In *Sox10*^{Dom}-homozygous mutant embryos, neural crest cells fail to colonize the gut and instead undergo massive cell death (22). Similarly, the analysis of *Sox10*$^{lacZ/lacZ}$ embryos revealed a requirement of *Sox10* for survival of neural crest cells (24) and, consistently, enteric progenitors are completely absent in the gut of *Sox10*$^{lacZ/lacZ}$ embryos (19). Thus, the loss of enteric progenitors in *Sox10*$^{lacZ/+}$ embryos might be attributed to increased apoptosis of mutant cells. To investigate this issue, we performed a TUNEL assay on gut sections at various developmental stages. Comparison of wild-type and *Sox10*-heterozygous mutant embryos at E11, E12, E13 and E15 revealed generally low numbers of apoptotic figures in the embryonic gut and did not indicate any increased cell death of enteric cells in mutant intestine (Fig. 4; and data not shown). These data are consistent with our previous finding, that *Sox10* homozygous but not heterozygous mutation affects survival of cultured multipotent trunk neural crest cells (24). Cell death, therefore, cannot be responsible for the reduced amount of enteric progenitor cells found in *Sox10*-heterozygous mutants.

Enteric neural crest-derived cells lose their progenitor state and acquire preneuronal traits in *Sox10*-heterozygous mutant animals

Cell culture experiments had allowed us to demonstrate that, dependent on the extracellular context, *Sox10*$^{lacZ/+}$ trunk neural crest cells adopt fates different from those chosen by their wild-type counterparts (24). Accordingly, the phenotype in the ENS of *Sox10*-heterozygous mutant animals might also be explained by aberrant cell fate decisions. In the *Sox10*-mutant mice we used, the complete open reading frame of *Sox10* has been replaced by *lacZ* gene sequences (19). Therefore, *Sox10* promoter activity can be monitored in *Sox10*^{lacZ} mice by measuring β-galactosidase activity. Thereby, the persistence of this enzyme in enteric neural crest derivatives not only allows detection of cells that express *Sox10* but also *in vivo* fate mapping of cells in which the *Sox10* promoter has been active at earlier stages. To follow the fate of mutant cells, we performed β-galactosidase stainings on sections of *Sox10*$^{lacZ/+}$ embryos at E13 and E15. In parallel, *in situ* hybridization experiments using a Sox10 riboprobe were carried out on adjacent sections of mutant embryos and on wild-type sections displaying gut segments comparable to the mutant. As mentioned above, the mutant hindgut lacked Sox10-positive cells at E13 while Sox10 expression was found in the wild-type hindgut. Likewise, β-galactosidase activity was undetectable in the hindgut of *Sox10*-heterozygous mutants at E13 (Fig. 5C, inset). Given the absence of cell death at all stages examined (Fig. 4; and data not shown), our data suggest that neither Sox10-expressing progenitor cells nor their derivatives were able to properly colonize the hindgut.

In the midgut, Sox10 expression in mutant embryos was reduced at E13 as compared to the wild-type (Fig. 5A and B; see also Fig. 2). This reduction was even more obvious at E15, when in some areas of the mutant midgut Sox10 expression was below detection limits while robust expression was found in similar areas of wild-type embryos (Fig. 5D and E). Strikingly, both at E13 and E15, large numbers of cells in the enteric plexus of *Sox10*$^{lacZ/+}$ mutant midgut displayed β-galactosidase activity even in regions in which Sox10 was not or was barely detectable on adjacent sections (Fig. 5B, C, E and F). β-galactosidase activity persisted at least up to E17, the latest stage examined (Fig. 6I; and data not shown). Thus, descendents of Sox10-expressing cells had localized in high numbers to midgut portions of *Sox10*-heterozygous embryos but, unlike many of their wild-type counterparts, down-regulated expression of the progenitor state marker Sox10. This finding was supported by the quantitative analysis of β-galactosidase-positive cells obtained by dissociating whole intestine of mutant embryos. While at E17 only 3.5% of all cells were marked by Sox10 immunostaining (Fig. 3G), ~11% of all cells displayed β-galactosidase activity, indicating that most neural crest-derived cells in the mutant had lost Sox10 protein expression.

The failure of neural crest-derived cells to maintain their progenitor state raises the question of what fate these cells

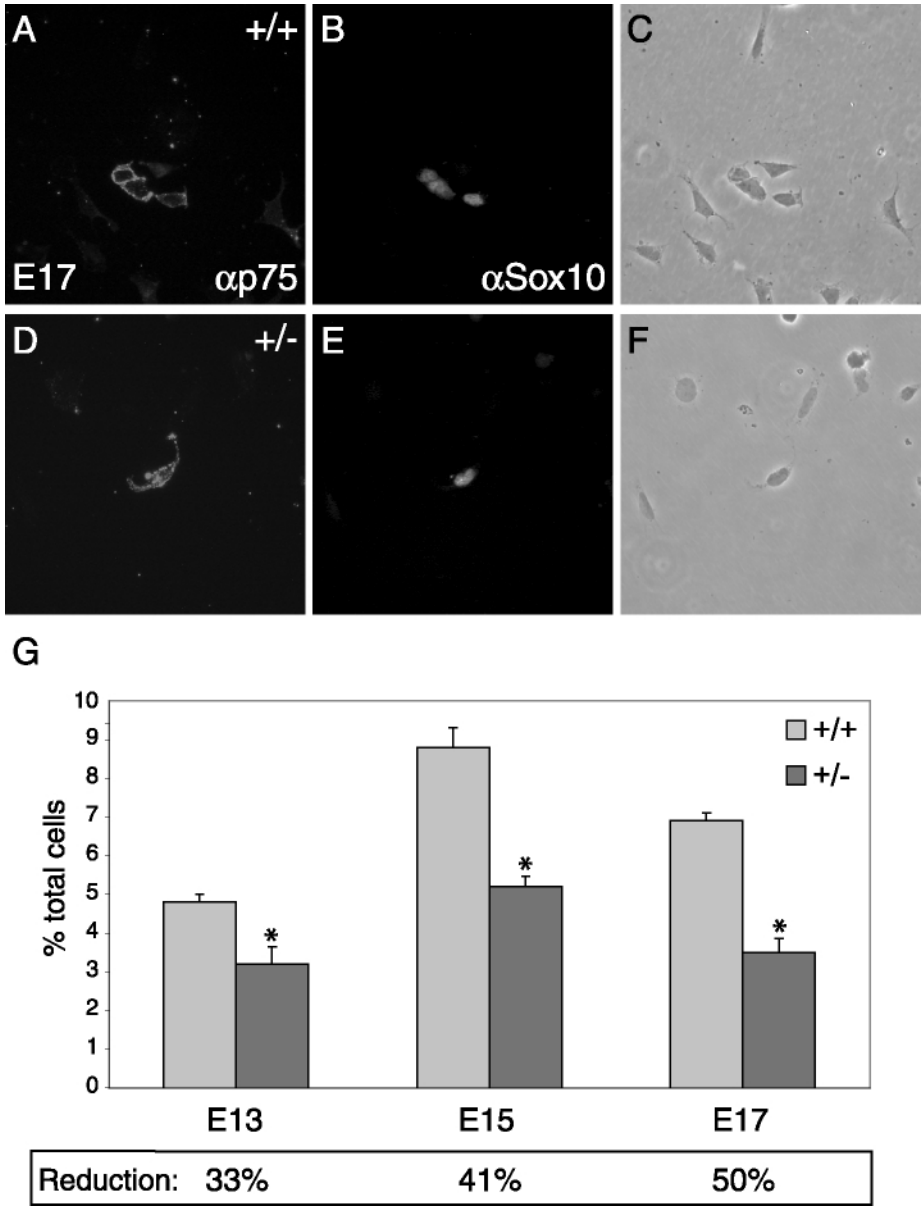

Figure 3. Reduction of the ENS progenitor pool in *Sox10*$^{lacZ/+}$ mutants. The intestine of wild-type and mutant animals was dissected, dissociated and plated onto cell culture dishes. The cells were immunolabeled for both p75 (visualized by a Cy3-coupled secondary antibody) (**A**, **D**) and Sox10 (visualized by FITC) (**B**, **E**). (**C**) and (**F**) show corresponding phase pictures. (**G**) The fraction of cells that were progenitors was determined by anti-p75/anti-Sox10 double immunocytochemistry. The numbers represent mean values from three independent animals, counting ∼1000 cells each. *Sox10*$^{lacZ/+}$ mutants showed a significantly reduced progenitor pool at stages E13, E15 and E17. A Student's *t*-test revealed significant differences between control and mutant animals: E13, $P < 0.003$; E15, E17, $P < 0.0001$.

adopt instead. Previously we have demonstrated that aberrant fate decisions by *Sox10*-heterozygous mutant trunk neural crest cells are context-dependent (24). In particular, when cell–cell interactions are allowed to occur in the absence of instructive gliogenic growth factors, these mutant cells are biased to generate neuronal cells. Alternatively, mutant cells might adopt a non-neuronal fate. During ENS development, the tyrosine kinase receptor c-Ret is first expressed by postmigratory enteric progenitor cells derived from p75/Sox10/ErbB3-positive neural crest cells and is later confined to the neuronal lineage (33,34). *In vitro*, Sox10 regulates the expression of *c-ret* in synergy with the transcription factor Pax3 (35) and mutations in *Sox10* might thus prevent expression of *c-ret* and other preneuronal traits. To determine the nature of the enteric cells derived from mutant progenitor cells, we performed double labeling experiments, staining midgut sections of E15 embryos for β-galactosidase and early neuronal markers. Confocal microscopy analysis of midgut sections at E15 revealed that virtually all mutant neural crest-derived cells expressing β-galactosidase also expressed c-Ret (Fig. 6A–C). The neuron-specific protein PGP9.5 is expressed in enteric preneuronal and neuronal cells that have downregulated Sox10 protein expression (25,36,37). Strikingly,

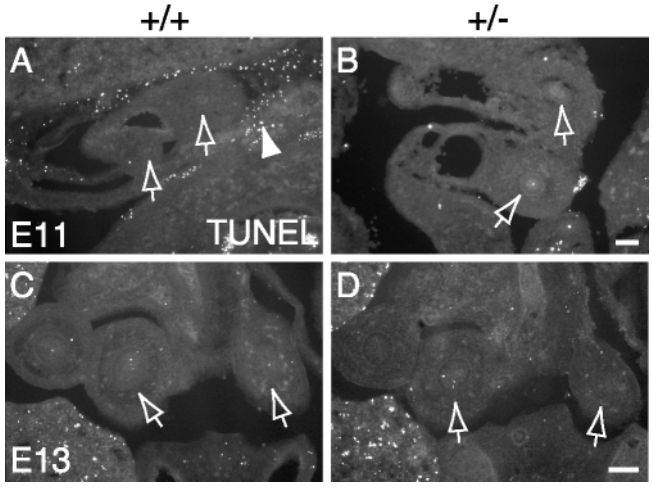

Figure 4. *Sox10* haploinsufficiency does not affect survival of ENS cells. TUNEL assays on gut sections at developmental stages E11 and E13 did not indicate any increased cell death in the gut of heterozygous (**B**, **D**; arrows) as compared with wild-type (**A**, **C**; arrows) sections. The arrowhead in (A) indicates apoptotic cells in areas outside of the gut. Scale bars: (B) 200 μm; (D) 400 μm.

the vast majority of β-galactosidase-positive cells found in $Sox10^{lacZ/+}$ mutant midgut coexpressed PGP9.5 (Fig. 6D–F). In contrast, cells expressing late neuronal differentiation markers such as neurofilament 160 and peripherin were detectable much less frequently than β-galactosidase-expressing cells, both at E15 and E17 (Fig. 6G–I; and data not shown). Thus, without leading to precocious appearance of fully differentiated neurons, *Sox10* haploinsufficiency promotes loss of the progenitor state and preferential acquisition of neuronal precursor characteristics by mutant neural crest cells in the gut.

DISCUSSION

Mutations in *Sox10* have been associated with enteric aganglionosis both in mice as well as in human patients with Hirschsprung disease (1,38). However, the molecular and cellular basis underlying the etiology of this disease has been elusive. In this study, we present evidence that aberrant fate decisions by enteric progenitor cells very early in development result in the absence of enteric ganglia in the distal gut of afflicted individuals (Fig. 7). In wild-type embryos, vagal neural crest cells, the major source of the ENS, migrate into proximal portions of the gut to gradually colonize its entire length (1). Although neurogenesis is observed at early developmental stages, progenitor cells with migratory capacity are maintained during prolonged periods of development (32). In a mouse model of Hirschsprung disease, *Sox10*-heterozygous mutant neural crest cells are able to migrate into proximal portions of the intestine during early embryonic development. However, in contrast to wild-type cells, mutant cells progressively lose their progenitor state and instead adopt traits of neuronal precursors. This results in a depletion of the overall progenitor pool. As a consequence, the hindgut in the mutant cannot be colonized by progenitor cells, leading to aganglionosis of the terminal bowel.

Context-dependent fate decisions by *Sox10*-mutant neural crest cells

Previously, it has been shown that most neural crest derivatives are defective in mice homozygous for the *Dom* mutation or for a targeted deletion of *Sox10* (17–19). In particular, the entire ENS is missing in these mutants, as neural crest cells completely fail to colonize the gut. This phenotype was explained by death of neural crest cells at early stages of neural crest development (22). Accordingly, multipotent trunk neural crest cells isolated from $Sox10^{lacZ/lacZ}$ mice display decreased survival capacity, revealing a cell-autonomous role of *Sox10* in survival (24). The question was raised, therefore, whether aganglionosis in the distal gut of *Sox10*-heterozygous mutants might also be due to increased death of neural crest cells (39). However, the survival function of *Sox10* can apparently be elicited by Sox10-protein levels present in heterozygous mutant cells, since cultured *Sox10*-heterozygous mutant neural crest cells, unlike homozygous mutant cells, survive as well as wild-type neural crest cells (24). In accordance with this finding, cell death was not increased in the developing ENS of $Sox10^{lacZ/+}$ animals, as assayed by TUNEL at several developmental stages.

Analysis of the developmental potential of wild-type and mutant trunk neural crest cells revealed, however, a second function of *Sox10* in multipotent neural crest cells. Under certain conditions, mutant cells tend to acquire aberrant fates as compared with wild-type cells, depending on the presence or absence of instructive growth factors and on short-range cell–cell interactions (24). These findings demonstrate that changing Sox10 levels in multipotent neural crest cells alters their responsiveness to the multiple cues present in the extracellular environment. In the developing embryo, migratory and postmigratory mutant neural crest cells are obviously exposed to different environmental contexts that change with the localization of the cell and the developmental stage. In the context of the developing gut, mutant progenitor cells are apparently unable to properly respond to their environment and to efficiently generate all ‘fates’ appropriate for early ENS development, namely maintenance of a progenitor state and adoption of a neuronal fate. This is best shown by *in vivo* fate mapping of *Sox10*-heterozygous mutant cells that express β-galactosidase from the *Sox10* locus. The extent of β-galactosidase activity in proximal segments of the mutant gut is comparable to the expression domain of neural markers in the wild type, confirming our above-mentioned data that the progeny of *Sox10*-expressing neural crest cells are not eliminated by cell death. However, most β-galactosidase-positive neural crest-derived cells fail to express the progenitor markers Sox10 and ErbB3 and the total number of undifferentiated enteric progenitor cells is substantially decreased in the mutant relative to the wild-type gut. Instead, we observed expression of early neuronal markers in the vast majority of mutant cells in the midgut. It is worth mentioning, though, that the total number of differentiated neurons in this area seems not to be altered as compared with wild-type embryos, indicating that neuronal cell numbers are regulated by secondary,

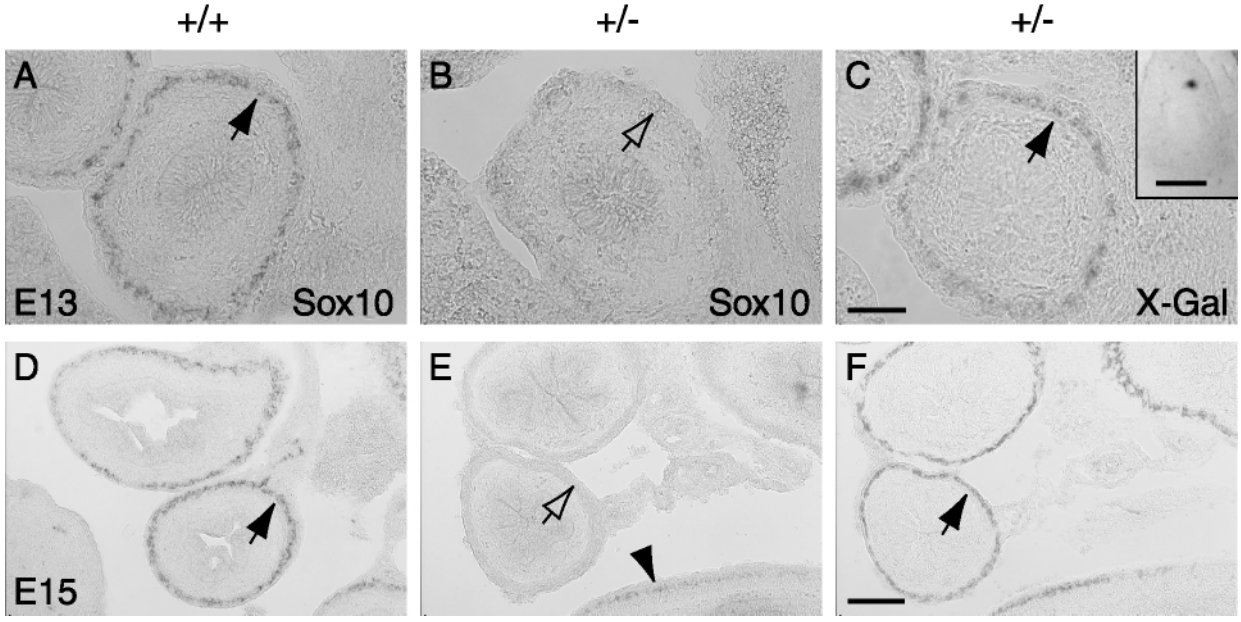

Figure 5. *In vivo* fate mapping of mutant ENS progenitor cells. *In situ* hybridization analysis on E13 and E15 midgut sections shows strong Sox10 staining in the wildtype (**A**, **D**; arrows). In contrast, at E13 Sox10 mRNA expression is barely detectable in the heterozygous midgut (**B**; open arrow) and completely gone at E15 (**E**; open arrow). Note the readily detectable Sox10 expression in more proximal regions of the mutant gut (E, arrowhead). (**C**, **F**) In the mutant, β-galactosidase is expressed from the *Sox10*-locus. Persistence of enzyme activity allows fate mapping of mutant progenitor cells. Adjacent sections to (B) and (E) were stained for β-galactosidase activity and revealed prominent staining in the midgut at E13 (C; arrow) whereas the hindgut was devoid of β-galactosidase staining (inset in C). At E15, β-galactosidase staining was still present at sites (F; arrow) where Sox10 mRNA was not detectable (E, open arrow), indicating that cells derived from progenitors have survived but downregulated Sox10 expression. Scale bars: (C) 200 μm; inset in (C) 100 μm; (F) 400 μm.

Sox10-independent mechanisms. In conclusion, we have shown that *Sox10* in neural crest cells is crucial for the maintenance of the progenitor state in the developing ENS. The behavior of isolated *Sox10*-mutant neural crest cells in culture and the fact that mutant gut is permissive to colonization by wild-type ENS progenitors indicates that this function of *Sox10* in ENS progenitors is cell-autonomous (22,24).

Sox10-mutant mice as a model system for Hirschsprung disease

Our finding that the enteric progenitor pool is not maintained in *Sox10*-mutant embryos raises the question of how this leads to the observed aganglionosis in the hindgut. It has been proposed that the migratory ability of enteric crest-derived cells decreases with their progressive differentiation, with neuroblasts and neurons being more sessile than undifferentiated progenitor cells (40). Accordingly, the generation of neuronal cells with the concomitant loss of the progenitor state in *Sox10*-heterozygous neural crest cells would result in a depletion of cells with migratory potential. Alternatively, the depletion of progenitor cells might simply lead to a decrease in total cell numbers of the mutant ENS. In both cases, the hindgut becomes aganglionic because it is the last part of the gut to be colonized by neural crest-derived cells (41). PGP9.5 and c-Ret, which we show to be preferentially expressed by *Sox10*-heterozygous mutant cells, are detectable at early stages of normal ENS development and are not only marking committed or even differentiated neuronal cells (33,36,37,42). Although the migratory capacity of c-Ret/PGP9.5-positive cells has not been directly compared with that of p75/Sox10-double positive and c-Ret-negative ENS-derived neural crest cells, c-Ret-positive cells are able to colonize both wild-type and aganglionic gut (42). This might suggest that it is not a decreased migratory capacity of mutant cells *per se* that would result in an aganglionic terminal bowel in Hirschsprung disease. However, the developmental potential of c-Ret-expressing cells appears to be more restricted than the potential of p75-positive/c-Ret-negative neural crest cells, with some c-Ret-positive cells being already committed to a neuronal fate (33). Most importantly, c-Ret-positive ENS cells display a decreased proliferative capacity as compared with p75-positive/c-Ret-negative neural crest cells (33). This is true even for uncommitted c-Ret-positive cells that quickly segregate into neuronal and non-neuronal lineages. Based on this we propose (Fig. 7) that by generating c-Ret-positive cells at the expense of c-Ret-negative neural crest-derived progenitor cells, the proliferation rate of ENS cells is reduced in *Sox10*-heterozygous mutants. The decreased progenitor pool size described in this study can thus not be compensated by the cells aberrantly generated in the mutant, preventing efficient colonization of distal gut segments by neural crest-derived cells.

In the wild-type, two sources of neural crest cells contribute to colonization of the terminal bowel, vagal and sacral neural crest (1). Vagal neural crest gives rise to ENS cells along the entire length of the gut, whereas sacral neural crest cells only colonize hindgut portions. The virtually complete absence of neural crest-derived cells in *Sox10*-mutant hindgut at E13 as assayed by Sox10-, ErbB3-, β-galactosidase- and neuronal marker expression suggests that, apart from vagal neural crest cells, sacral cells are also affected by the *Sox10* mutation.

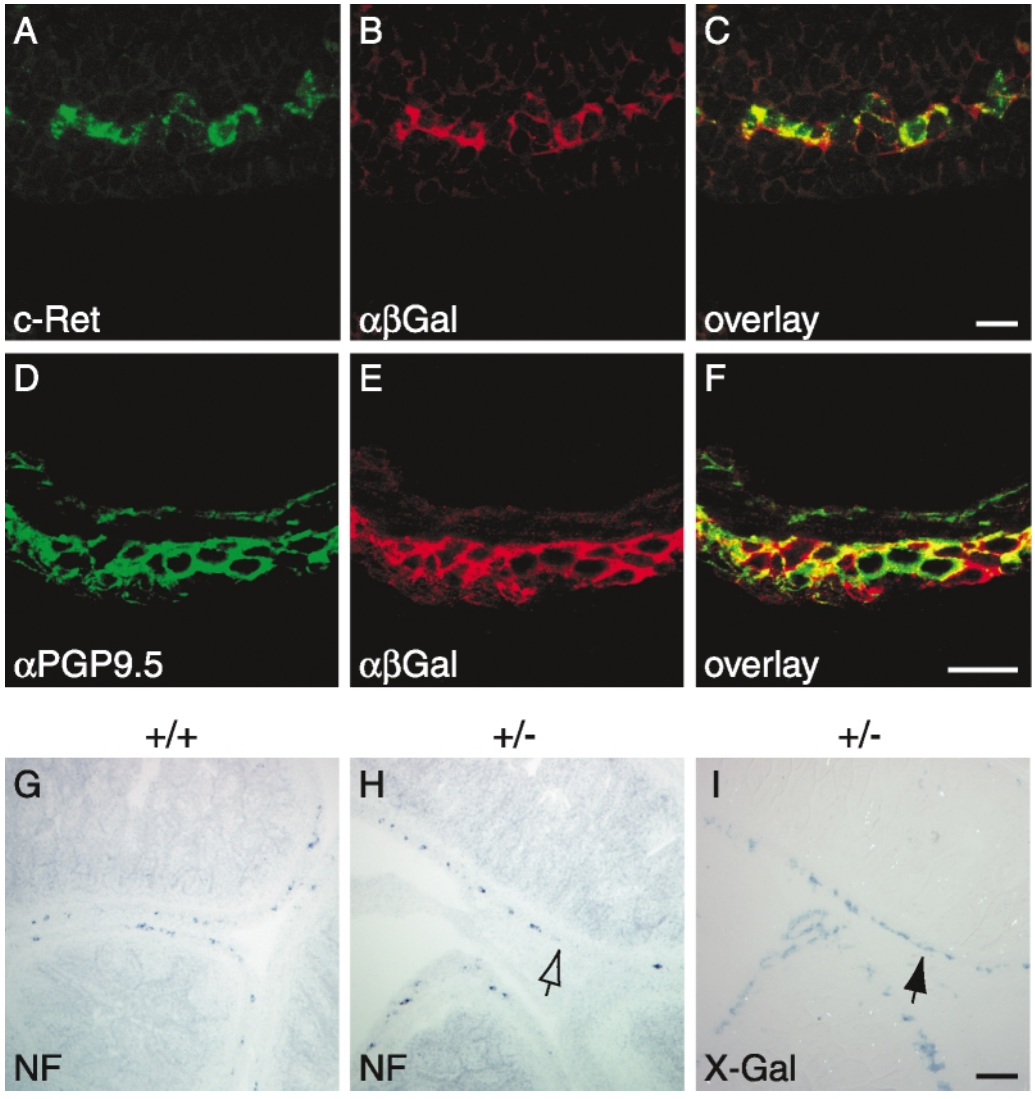

Figure 6. β-Galactosidase-positive cells acquire early neuronal traits in the *Sox10*$^{lacZ/+}$ mutant ENS. Confocal microscopy analysis of midgut sections at E15 revealed that many cells that are c-Ret positive as assessed by fluorescence *in situ* hybridization (**A**) are also positive for anti-β-galactosidase immunostaining (**B**). Moreover, the anti-β-galactosidase staining (**E**) co-localizes with the neuronal marker PGP9.5 in many cells (**D**). (**C**) and (**F**) represent the corresponding confocal overlays; the yellow color indicates double-positive cells. Single confocal planes are shown. *In situ* hybridization on E17 gut sections revealed relatively few NF-positive differentiated neurons in the wild type (**G**) and the mutant (**H**). The domain of β-galactosidase staining on mutant midgut sections (**I**; arrow) is broader than the NF-expression pattern (**H**; open arrow). The section shown in (I) is adjacent to the section displayed in (H). Scale bars: (C, F) 20 µm; (I) 40 µm.

The phenotype described in this study can, however, not be explained solely by a failure in sacral neural crest cell development, as progenitors in the midgut (which are derived from vagal crest) are evidently affected. Moreover, it has been shown that sacral neural crest cells only form a minority of the postumbilical ENS that cannot compensate for missing vagal crest-derived cells (41,43,44). Thus, we propose that the loss of vagal crest-derived progenitor cells that is apparent in relatively proximal parts of the gut is the main cause for the absence of an ENS in more distal gut segments (Fig. 7).

Depletion of the enteric progenitor pool might also be the cellular basis underlying Hirschsprung disease in patients with mutations in genes of the ET-3/ET_B signal transduction pathway (8). Signaling by ET-3/ET_B has an inhibitory effect on the differentiation of enteric neurons (36,45). Therefore, deficiencies in ET-3/ET_B-signaling are thought to lead to premature neuronal differentiation and, as a consequence, to affect the progenitor pool size. Unlike in *Sox10*-mutants, however, the resulting aganglionosis in the terminal bowel of *ET-3/ET$_B$* mutants cannot be explained solely by a neural crest cell-autonomous effect, since an abnormal gut environment contributes to the enteric phenotype in mice deficient in ET-3/ET_B-signaling (40). Mutations in *RET* and in genes encoding the growth factors GDNF and NTN that signal through c-Ret also lead to Hirschsprung disease (3). Although it has been suggested that some mutations cause c-Ret to constitutively induce apoptosis (46), the cellular mechanisms leading to aganglionosis in the terminal bowel of patients with *GDNF/NTN/RET* mutations is unclear. Mouse models demonstrate a crucial role of *GDNF/NTN/c-ret* in ENS development but, unlike

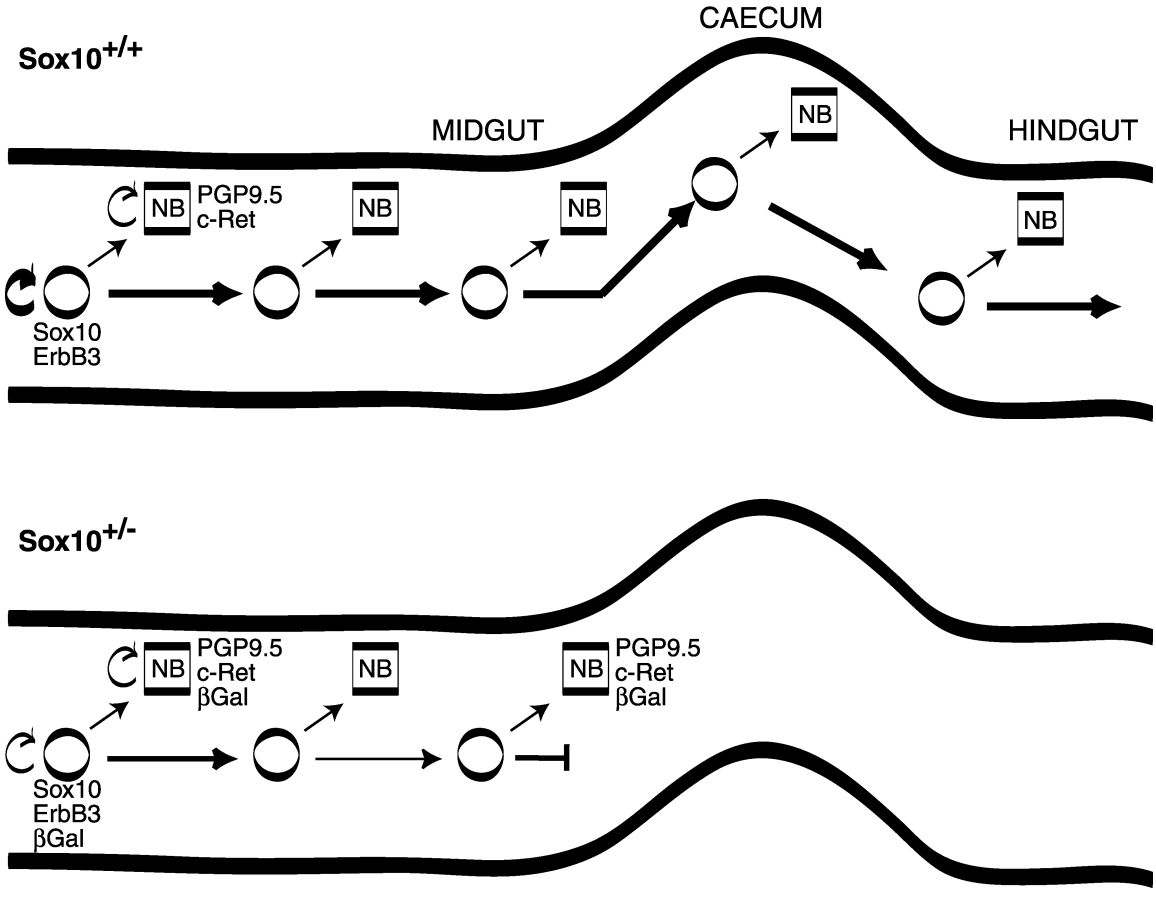

Figure 7. Cellular basis for Hirschsprung disease in *Sox10*$^{lacZ/+}$ animals. During normal enteric development, p75^{+}/Sox10^{+}/ErbB3^{+} vagal neural crest cells migrate into the proximal part of the gut and gradually colonize the entire length of the gut to give rise to differentiating neuroblasts (NB) that express the neuronal precursor markers c-Ret and PGP9.5. In *Sox10*$^{lacZ/+}$ mutants, maintenance of the progenitor state is impaired (illustrated by thinner arrows) and, therefore, the number of progenitor cells produced in the gut is reduced. Cells expressing c-Ret and PGP9.5 are generated at the expense of p75^{+}/Sox10^{+}/ErbB3^{+} neural crest-derived progenitor cells. These preneuronal cells can be detected by virtue of the β-galactosidase activity persisting in neural crest-derived cells. They have a reduced proliferative capacity (33) and can therefore not compensate for the loss of undifferentiated neural crest-derived progenitors in the mutant. As a consequence, cells with migratory capacity are not present in high enough numbers to colonize more distal parts of the gut. The absence of progenitor cells in the hindgut ultimately leads to aganglionosis of the terminal bowel.

heterozygously-affected human patients, only display a phenotype as homozygotes in which not only the distal gut but the entire intestine is affected (3,4). Nonetheless, given the mitogenic activity of GDNF on neural crest-derived enteric progenitors and the massive cell death of ENS progenitors in *c-ret*-null mice, it is conceivable that aganglionosis in patients with *GDNF/NTN/RET* mutations results from decreased proliferation of progenitors or increased cell death or both of these (31,45,47).

In human patients, none of the mutations discussed above are fully penetrant, identifying Hirschsprung disease as a multifactorial disorder (1,48). Interactions between different genetic loci, including modulation of an ET_B-dependent phenotype by *RET* variants, have been reported. Variability in penetrance and disease phenotype has also pointed to the existence of *SOX10*-dependent modifier genes (20,21). To further elucidate the etiology of Hirschsprung disease, it will be important to identify these modifier genes (48) and to investigate the cellular consequences of their interactions with known Hirschsprung disease genes. The mouse mutant used in the present study is an ideal model system to address such issues, given that, as in human patients, mutations in *Sox10* are haploinsufficient and pleiotropic in mice. With this system it should be feasible to determine the molecular mechanisms by which Sox10 regulates maintenance of ENS progenitors and how this process might be modulated by other factors.

MATERIALS AND METHODS

Sox10^{lacZ} mice

Sox10-mutant mice were maintained in a C3HeB/FeJ background (19). All experiments were performed analysing the F1 generation by crossing mutant mice with C57/Bl6 mice. Tissue from a forelimb was used for genotyping by PCR (19).

Cell cultures

Cell culture preparations of dissociated gut were done as follows: at E13, the entire gut distal to the stomach was

dissected and digested in 1 mg/ml collagenase type I (Worthington Biochemical, NJ, USA) in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (Amimed) for 20 min at 37°C. Preparations from E15 and E17 were digested for 45 min in a solution that, in addition to the collagenase, contained 0.01% trypsin (Gibco BRL). The digestion was stopped by the addition of 10% fetal bovine serum (FBS). Subsequently, the cells were centrifuged for 2 min at 680 *g*, triturated, and plated onto dishes coated with 0.25 mg/ml fibronectin (Roche Diagnostics). Four hours after plating in standard medium (24), the cells were fixed with 3.7% formaldehyde (FA) in phosphate-buffered saline (PBS) for 10 min and analysed.

Immuno- and β-galactosidase staining reactions

For immunocytochemistry, the cells were permeabilized and blocked for 10 min at room temperature (RT) with blocking buffer (10% goat serum, 0.3% Triton X-100, 0.1% BSA in PBS). Progenitor cells were labeled with rabbit anti-mouse p75 (Chemicon International) diluted 1 : 200 in blocking buffer for 1 h at RT and with a monoclonal anti-Sox10 antibody (1 : 3 dilution) (24) for 2 h at RT. Immunostaining on cryosections was performed with rabbit anti-β-galactosidase (ICN/Kappel) diluted 1 : 100 in blocking buffer for 2 h at RT and monoclonal anti-human PGP9.5 (UltraClone) diluted 1 : 40 for 3 h at RT. All immunostainings were visualized by incubation for 1 h at RT using the following reagents at a 1 : 200 dilution: Cy3-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Jackson Immuno Research Laboratories); and FITC-conjugated horse anti-mouse IgG (Vector Laboratories). For X-Gal staining on cryosections, the sections were fixed for 10 min in 2% FA at RT, washed twice in PBS and stained for 2 h at 37°C in staining solution (1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$; 5 mM $K_4Fe(CN)_6$; 2 mM $MgCl_2$ in PBS).

TUNEL assay

Cryosections were fixed in 2% PFA/PBS for 10 min, washed three times for 5 min in PBS and blocked for 1 h in 10% goat serum, 0.1% BSA, 1% Triton X-100 in PBS. The TUNEL staining was performed according to the manufacturer's instructions (Roche Diagnostics) and visualized by Cy3-conjugated streptavidin (1 : 200 dilution, Jackson ImmunoResearch Laboratories). Finally, the sections were mounted in AF1 (Citifluor).

Non-radioactive *in situ* hybridization

Non-radioactive *in situ* hybridization with digoxigenin-labeled riboprobes was performed on frozen sections of paraformaldehyde-fixed mouse embryos as described in (49). NBT/BCIP were used as chromogens to visualize the hybridization signals. The c-Ret fluorescent *in situ* hybridization combined with anti-β-galactosidase immunostaining was done as reported in (49) using the tyramide signal amplification method (Perkin Elmer). After washing in PBS, the sections were mounted in AF1 (Citifluor) and analysed by confocal microscopy.

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