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Notch signaling is required for the maintenance of enteric neural crest progenitors

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Notch signaling is involved in neurogenesis, including that of the peripheral nervous system as derived from neural crest cells (NCCs). However, it remains unclear which step is regulated by this signaling. To address this question, we took advantage of the Cre-loxP system to specifically eliminate the protein *O*-fucosyltransferase 1 (*Pofut1*) gene, which is a core component of Notch signaling, in NCCs. NCC-specific *Pofut1*-knockout mice died within 1 day of birth, accompanied by a defect of enteric nervous system (ENS) development. These embryos showed a reduction in enteric neural crest cells (ENCCs) resulting from premature neurogenesis. We found that Sox10 expression, which is normally maintained in ENCC progenitors, was decreased in *Pofut1*-null ENCCs. By contrast, the number of ENCCs that expressed Mash1, a potent repressor of Sox10, was increased in the *Pofut1*-null mouse. Given that Mash1 is suppressed via the Notch signaling pathway, we propose a model in which ENCCs have a cell-autonomous differentiating program for neurons as reflected in the expression of Mash1, and in which Notch signaling is required for the maintenance of ENS progenitors by attenuating this cell-autonomous program via the suppression of Mash1.

KEY WORDS: Neural crest cells, Pofut1, Sox10, Mash1 (Ascl1), Wnt1-Cre

INTRODUCTION

The neural crest is a pluripotent cell population derived from the neural plate during the early stages of embryogenesis. Neural crest cells (NCCs) disperse from the dorsal surface of the neural tube and migrate extensively throughout the embryo. This gives rise to a wide variety of differentiated cell types, including neurons, glial cells, pigment cells, facial cartilage and bone, and connective tissues (Le Douarin et al., 2004). The fate of individual NCCs largely depends upon the locations to which they migrate. Some NCCs contribute to the enteric nervous system (ENS), which yields neurons and glial cells. The ENS arises from vagal and sacral NCCs.

At E8.5, the vagal NCCs detach from the dorsal neural tube at somite level 1-7, colonize the foregut at E9.5, then migrate in a caudal direction and reach the rectum by E14 (Young et al., 1998). Sacral NCCs are delaminated from the neural tube that is posterior to somite 28. Some of these cells give rise to pelvic ganglia, whereas others contribute to the ENS by colonizing the hindgut in a caudal-rostral migratory path (Druckenbrod and Epstein, 2005; Kapur, 2000).

The ENS constitutes a part of the autonomic nervous system that regulates peristalsis, secretion, blood supply and the immune response in the intestinal tract. Defects in neural crest development are a significant cause of Hirschsprung disease in humans, which is characterized by distal intestinal aganglionosis and which occurs about once in every 5000 live births (Swenson, 2002). Many genes, including *Edn3*, *Ednrb*, *Sox10*, *Ret*, *Gfra1* and *Gdnf*, have been implicated in the pathogenesis of congenital megacolon, and these genes control the development of the ENS through their coordinated activities (Barlow et al., 2003; Baynash et al., 1994; Enomoto et al., 1998; Herbarth et al., 1998; Hosoda et al., 1994; Schuchardt et al., 1994; Stanchina et al., 2006). During the development of the ENS, the transcriptional regulator Sox10 is essential for the maintenance of neural crest progenitors (Paratore et al., 2002). The expression of Sox10, a member of the SRY-like HMG-box family of transcription factors, is initiated in NCCs when they detach from the neural tube and is maintained during NCC migration. Eventually, Sox10 expression is turned off in the NCC derivatives, except for the glial and melanocyte lineages (Kuhlbrodt et al., 1998; Pusch et al., 1998). Two mutations of *Sox10* have been examined in mice: a null mutation generated by targeted insertion of *lacZ* (*Sox10*^{lacZ}), and a spontaneous mutation referred to as *Dominant megacolon* (*Sox10*^{Dom}) (Britsch et al., 2001; Herbarth et al., 1998; Southard-Smith et al., 1998). In the heterozygous state, both mutants lack enteric ganglia in a variable length of the colon and exhibit pigmentation defects. Homozygous mutants display severe deficits in several neural crest derivatives, including complete absence of the ENS (Britsch et al., 2001; Kapur, 1999). By contrast, the overexpression of *Sox10* in neural crest stem cells (NCSCs) preserves both glial and neuronal differentiation potentials (Kim et al., 2003).

In addition to these mutants, it is known that *Mash1* (*Ascl1* – Mouse Genome Informatics) mutant mice also show defects in ENS development. *Mash1* encodes a member of the basic helix-loop-helix family of transcriptional regulators and is a mammalian homolog of the *Drosophila* proneural genes of the *achaete-scute* complex; *Mash1* is expressed in both the central nervous system (CNS) and the peripheral nervous system and is induced by bone morphogenetic protein 2 (Bmp2) in NCSCs (Shah et al., 1996). *Mash1* mutant mice show defects in the development of olfactory and peripheral autonomic neurons and a loss of part of the enteric neuron lineages (Blaugrund et al., 1996; Guillemot et al., 1993).

Notch is one of the transmembrane receptors activated by the juxtacrine interactions of specific ligands with Notch receptors (Artavanis-Tsakonas et al., 1999). Once activated, the Notch receptors then undergo a series of proteolytic cleavages, resulting in the release of the Notch intracellular domain (NICD) (Mumm et al., 2000). Subsequently, the NICD translocates into the nucleus, associates with the transcription factor RBP-Jκ (Rbpj – Mouse

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Genome Informatics) to generate the transactivation complex, and thereby initiates the transcription of target genes such as *Hes1* (Kageyama et al., 2000). In addition to the four Notch receptors (Notch1-4) and five ligands (delta-like 1, 3, 4 and jagged 1, 2) that have been identified in vertebrates, there are many auxiliary factors through which Notch signaling is tightly regulated (Bray, 2006). One of these, protein *O*-fucosyltransferase 1 (Pofut1), which transfers *O*-fucose to Notch EGF repeats, is an essential component of Notch signaling and functions upstream of NICD, where it regulates Notch-Delta interactions and trafficking of the Notch receptor (Okajima and Irvine, 2002; Okajima et al., 2003; Okajima et al., 2005; Okamura and Saga, 2008; Sasamura et al., 2007; Sasamura et al., 2003; Shi and Stanley, 2003). Although Pofut1 is ubiquitously expressed in the mouse, its removal leads to global Notch signaling defects similar to those associated with the loss of RBP-Jk, the common mediator of Notch signaling in mice (Oka et al., 1995). This indicates that Pofut1 functions are largely restricted to the Notch signaling pathway.

Notch signaling is involved in various aspects of neurogenesis. In the CNS it is essential for the maintenance of neural stem cells (Hitoshi et al., 2002). Transient activation of Notch signaling is not sufficient to maintain NCSCs. Rather, it acts positively to promote switching from neurogenesis to gliogenesis in vitro (Morrison et al., 2000) and in vivo (Wakamatsu et al., 2000). In addition, Notch signaling regulates sympathetic neuron development (Tsarovina et al., 2008), cardiac neural crest differentiation (High et al., 2007), melanocyte development (Moriyama et al., 2006) and mesencephalic neural crest differentiation (Ijuin et al., 2008). However, little is known regarding the role of Notch signaling in enteric neural crest development in vivo.

Here, we have examined the role of Notch signaling in the ENS by specifically eliminating the *Pofut1* gene in NCCs. These *Pofut1* conditional knockout (cKO) embryos showed a reduction in enteric neural crest cells (ENCCs) and premature neurogenesis, and this was accompanied by the loss of Sox10 expression and by an increase in the number of Mash1-positive ENCCs. These results suggest that Notch signaling is necessary for the maintenance of enteric neural crest progenitors via the regulation of Sox10 and Mash1 expression.

MATERIALS AND METHODS

Mice and genotyping

The generation of a conditional allele of the *Pofut1* locus has recently been described (Okamura and Saga, 2008). The generation of *CAG-CAT-EGFP*, *Wnt1-Cre* and *R26R* reporter mice has also been reported (Ahmed et al., 2002; Chai et al., 2000; Jiang et al., 2000; Soriano, 1999). Mouse tail or embryo yolk sac DNA was used for genotyping. Tissue samples were heated at 95°C for 5 minutes and then incubated in 100 mM Tris (pH 8), 5 mM EDTA, 300 μg/ml proteinase K at 55°C overnight. After heat denaturation at 95°C for 5 minutes, tissue suspensions were used as PCR templates. PCR primers used for genotyping were as follows: *Pofut1* mice, LA-4.9 (5′-CCATTGTGCGGTGCATTGTG-3′), cprb-L1 (5′-GCACTCTGGGGCT-CTGCCGT-3′) and SA-R0.01 (5′-CTGTACCCAGGCAAGTAGGG-3′); *Wnt1-Cre* mice, Cre1 (5′-GGACATGTTCAGGGATCGCCAGGCG-3′) and Cre2 (5′-GCATAACCAGTGAAACAGCATTGCTG-3′); Rosa26 reporter, Rosa-2 (5′-GCGAAGAGTTTGTCCTCAACC-3′), Rosa-3 (5′-GGAGCGGGAGAAATGGATATG-3′), Rosa-4 (5′-AAAGTCGCTCTGA-GTTGTTAT-3′); CAG-CAT-EGFP mice, CAT2 (5′-CAGTCAGTTGC-TCAATGTACC-3′), CAT3 (5′-ACTGGTGAAACTCACCCA-3′).

Histological analyses

Whole-mount X-Gal staining of embryos was as described (Saga et al., 1992). The number of X-Gal-positive ENCCs was counted and quantified as the number per unit area (100 μm^2). Whole-mount immunostaining using monoclonal anti-neurofilament antibody 2H3 (Developmental Studies Hybridoma Bank) was performed as described previously (Matsuo et al., 1995). For section immunohistochemistry, embryos were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C, cryoprotected in 30% sucrose in PBS at 4°C, embedded in OCT compound (Tissue-Tek) and sectioned (7 μm). The sections were blocked in PBS containing 10% normal donkey serum (Chemicon) for 1 hour at room temperature, then incubated overnight at 4°C in a mixture of the following primary antibodies in blocking solution: anti-GFP (Nacalai Tesque), anti-activated caspase 3 (Cell Signaling Technology), anti-β-tubulin isotype III (TuJ1) (Sigma), anti-BFABP (a gift from T. Müller, Max-Delbruck Center, Berlin, Germany), anti-Sox10 (Santa Cruz Biotechnology), anti-Hes1 (a gift from N. Brown, Cincinnati Children's Hospital Medical Center, Cincinnati, OH) and anti-Mash1 (BD Pharmingen). After washing, these were incubated with a mixture of Alexa Fluor 488- and 594- (Molecular Probes) or Cy3- (Jackson Laboratories) conjugated secondary antibodies.

For BrdU labeling, pregnant mice were injected intraperitoneally with BrdU (Sigma) at 10 mg/kg body weight and sacrificed 2 hours later. Embryos were handled as described above and sectioned (7 μm). After blocking, these sections were incubated overnight at 4°C in anti-GFP or anti-Sox10 antibody in blocking solution. After washing, the samples were further incubated with Alexa Fluor 488-conjugated secondary antibody, washed in PBS and fixed in 4% PFA in PBS at 4°C. The specimens were then placed in 2M HCl for 30 minutes at 37°C, then washed in PBS. After blocking, the sections were incubated with anti-BrdU antibody (Sigma), washed in PBS and incubated with Cy3-conjugated secondary antibody.

For double staining using immunohistochemistry and in situ hybridization, frozen sections (12 μm) were incubated with anti-$p75^{NTR}$ primary antibody (1:200, Promega) for the detection of ENCCs, followed by a biotinylated goat anti-rabbit IgG secondary antibody (1:200, Vector Laboratories). These sections were then hybridized with digoxigenin (DIG)-labeled antisense cRNA probes (Roche). The hybridized probes were detected using horseradish peroxidase-conjugated anti-DIG sheep antibody (Roche) and Cyanine 3-Tyramid Signal Detection Reagent (Perkin Elmer). $p75^{NTR}$ was detected using horseradish peroxidase-conjugated Streptavidin (Roche) and fluorescein isothiocyanate-conjugated Tyramid signal detection (Perkin Elmer). Sections were counterstained with 0.5 μg/ml 4′,6-diamino-2-phenylindole (DAPI) for 10 minutes and examined using a scanning-laser confocal imaging system (Zeiss LSM510) or with an Olympus BX61 fluorescence microscope system with an ORCA-ER digital camera (Hamamatsu Photo). Subsequent analysis was performed using MetaMorph software (Universal Imaging). More than six sections from the stomach or intestine regions of three different embryos were counted in each experiment. Statistical analysis was performed using a Student's *t*-test. Differences were considered to be significant if the *P*-value was less than 0.05.

RESULTS

Targeted disruption of the *Pofut1* gene in mouse neural crest cells

To achieve a conditional deletion of the *Pofut1* locus in NCCs, we employed *Wnt1-Cre* transgenic mice that express Cre recombinase under the control of the *Wnt1* promoter. The Cre recombinase is active in the early migratory neural crest population of these mice at all axial levels (Chai et al., 2000; Jiang et al., 2000). We also utilized the Rosa26 reporter (*R26R*) and *CAG-CAT-EGFP*, which mark the Cre-expressed cell lineages via β-galactosidase and EGFP expression, respectively, after Cre-mediated excision of a floxed PGK-neo or CAT cassette. To generate a *Pofut1* deficiency in mouse NCCs, we crossed $Pofut1^{+/-}$; *Wnt1-Cre* mice with $Pofut1^{flox/flox}$ mice harboring a *R26R* or *CAG-CAT-EGFP* allele. From this cross, we obtained two genotypes: $Pofut1^{+/flox}$; *Wnt1-Cre* (*R26R* or *CAG-CAT-EGFP*), referred to hereafter as control; and $Pofut1^{-/flox}$; *Wnt1-Cre* (*R26R* or *CAG-CAT-EGFP*), referred to as *Pofut1* cKO.

The *Pofut1* cKO mice died within 1 day of birth, but showed no obvious morphological abnormalities other than a relatively small body size (Fig. 1A). However, 18/20 newborn *Pofut1* cKO mice had

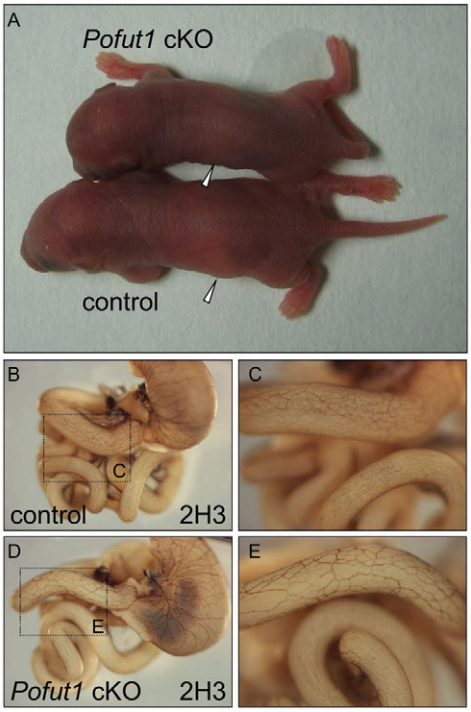

Fig. 1. Defects of the NCC-specific *Pofut1*-knockout mouse. (**A**) External morphology of control and *Pofut1* cKO mice at postnatal day 1 (P1). The *Pofut1* cKO mouse is relatively small, but otherwise shows no apparent morphological defects. Arrowheads indicate the stomach. (**B-E**) Immunostaining of the gastrointestinal tract at E16.5 in control (B,C) and *Pofut1* cKO (D,E) embryos with the anti-neurofilament antibody 2H3.

no milk in their stomachs (Fig. 1A, arrowheads). Since NCCs contribute to the formation of the ENS, we speculated that this phenotype might be due to defective ENS development. We examined enteric neuronal development in the gastrointestinal tract using anti-neurofilament antibody 2H3, which labels both extrinsic and intrinsic nerve fibers. The ganglion structure of the ENS was disrupted and dispersed and the nerve bundles in the *Pofut1* cKO embryos were relatively thick by comparison with the dense reticulate pattern of the enteric ganglia in the control embryos (Fig. 1B-E). These results suggest that Notch signaling participates in ENS development.

Embryos lacking *Pofut1* in NCCs show a reduction in post-migratory enteric neural crest cells

We examined enteric neural crest development by lineage tracing with *lacZ* in *R26R* reporter mice (Fig. 2). In control;*R26R* embryos, the ENCCs migrated to and colonized the foregut at around E9.5, then continued to migrate through the gut mesenchyme in a caudal direction, completing their migration at around E14 (Fig. 2A-D). Similar to control;*R26R* embryos, the ENCCs in *Pofut1* cKO;*R26R* embryos colonized the foregut and migrated to the hindgut by E11.5 (Fig. 2E,F). However, the number of ENCCs in these embryos was greatly reduced after E12.5 (Fig. 2G,H). Compared with control embryos, the number of ENCCs at E12.5 was estimated to be reduced to ~53% in the stomach, ~47% in the foregut and ~49% in the midgut and hindgut. Interestingly, specific regions in *Pofut1* cKO;*R26R* embryos showed a slightly elevated number of ENCCs as compared with other regions (see Fig. S1 in the supplementary material). In spite of their reduction in *Pofut1* cKO;*R26R* embryos, the ENCCs migrated on a normal timetable and contributed throughout the gastrointestinal tract by E14 (Fig. 2E-H), indicating that ENCC migration is not overtly disrupted by the loss of *Pofut1*. Hence, *Pofut1* cKO embryos exhibited an abnormal ENS network, for which a reduction in the number of ENCCs appeared responsible.

The decreased proliferation of ENCCs is partly responsible for their reduced number in *Pofut1* cKO embryos

To determine the underlying cause of the reduction of ENCCs in *Pofut1* cKO embryos, we examined the apoptotic and proliferation status of these cells (Fig. 3). For this analysis, we used a *CAG-CAT-EGFP* reporter to facilitate the immunohistological detection of ENCCs among other molecular markers. Apoptotic cells were not observed in control or *Pofut1* cKO ENCCs at E11.5 and E12.5 (Fig. 3A-H). This indicates that the loss of ENCCs in *Pofut1* cKO embryos is not due to an increase in apoptosis. However, our data do not exclude the possibility that non-apoptotic cell death contributes

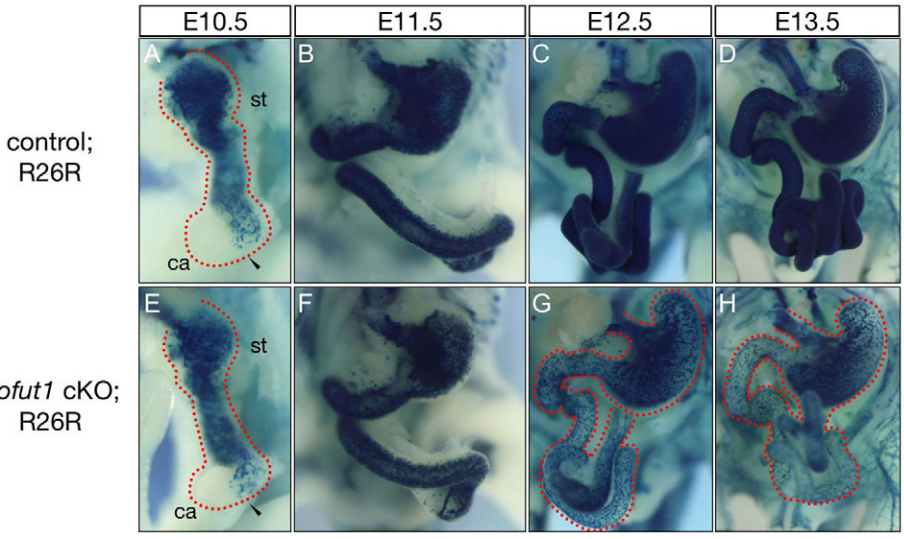

Fig. 2. Lineage analysis of ENCCs. Enteric neural crest lineages were traced using β-galactosidase activity from E10.5 to E13.5 in control (**A-D**) and *Pofut1*cKO (**E-H**) mouse embryos by crossing *Pofut1*$^{+/-}$; *Wnt1-Cre* and *Pofut1*$^{flox/flox}$;*R26R* mice. The gastrointestinal tracts are outlined (dashed lines). Arrowheads (A,E) indicate migratory wavefront of ENCCs. st, stomach; ca, cecum.

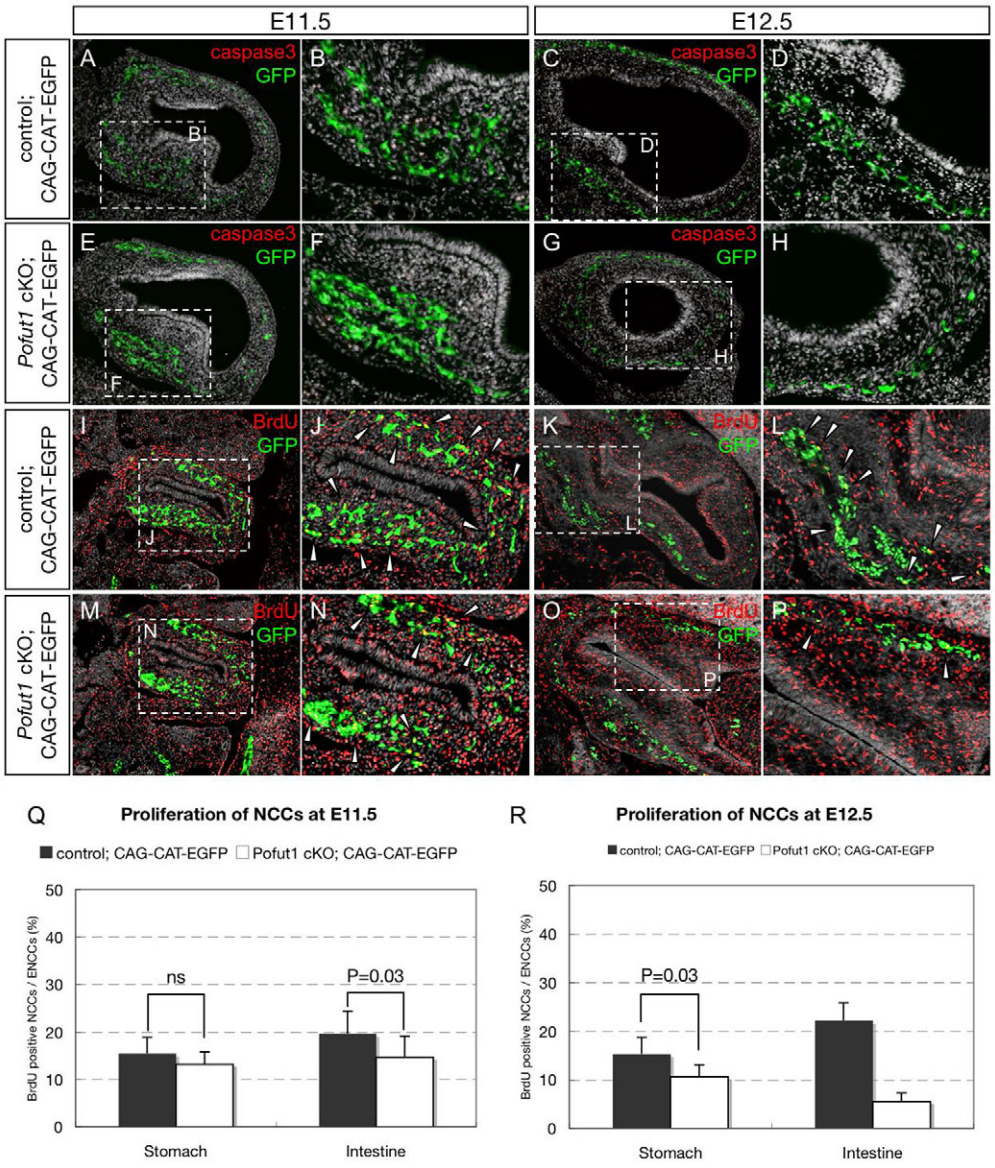

Fig. 3. Detection of apoptosis and proliferation in ENCCs. (**A-H**) Immunohistological analysis of control (A-D) and *Pofut1* cKO (E-H) mouse embryos using anti-caspase 3 (red) and anti-GFP (green) antibodies to visualize apoptotic cells and ENCCs, respectively, at E11.5 (A,B,E,F) and E12.5 (C,D,G,H). (**I-P**) Immunohistological analysis of control (I-L) and *Pofut1* cKO (M-P) embryos using anti-BrdU (red) and anti-GFP (green) antibodies to visualize proliferating cells and ENCCs, respectively, at E11.5 (I,J,,M,N) and E12 (K,L,O,P). Arrowheads indicate proliferating ENCCs. (**Q**,**R**) Quantitation of ENCC proliferation at E11.5 (Q) and E12.5 (R). Average percentages of BrdU-incorporating ENCCs are shown with s.d. ns, not significant.

to the loss of ENCCs, as it has recently been shown that the conditional ablation of *Gfra1* in mouse induces a loss of enteric NCCs via a caspase-independent mechanism (Uesaka et al., 2007).

We next examined proliferating cells in the gastrointestinal tract by BrdU-incorporation analyses at E11.5 and E12.5. The proliferation rate of ENCCs in *Pofut1* cKO embryos was found to be significantly reduced in the intestinal tract at E12.5 (Fig. 3I-R). Interestingly, consistent with the observation that slightly increased numbers of ENCCs exist in the stomach and cecum (see Fig. S1 in the supplementary material), the proliferating ENCCs in *Pofut1* cKO embryos at E12.5 were mainly located in the stomach and in the vicinity of the cecum, whereas these cells were uniformly detected throughout the gastrointestinal tract in control embryos (Fig. 3K,L,O,P; see Fig. S2 in the supplementary material). These regions are known to be sources of Gdnf and Edn3 (Burns and Thapar, 2006). Gdnf is known to promote the proliferation of ENS progenitors (Chalazonitis et al., 1998; Hearn et al., 1998; Heuckeroth et al., 1998; Taraviras et al., 1999), although the role of Edn3/Ednrb signaling remains controversial (Bondurand et al., 2006; Kruger et al., 2003). In either case, the localized proliferation we observed in *Pofut1* cKO embryos might well be influenced by such factors. Furthermore, despite their significant reduction in number, NCCs still contributed to development throughout the gastrointestinal tract in *Pofut1* cKO embryos. This is likely to be due to the presence of a few proliferating ENCCs. Our results thus suggest that the loss of ENCCs in *Pofut1* cKO embryos might be partially, although not solely, due to a decrease in their proliferation.

ENCCs lacking *Pofut1* show premature neurogenesis and a decrease in the number of glial progenitors

Since the decreased number of ENCCs in *Pofut1* cKO embryos could also be explained by premature differentiation, we examined the differentiation status of ENCCs in these embryos using the post-mitotic neuron marker TuJ1 (Tubb3 – Mouse Genome Informatics) (Fig. 4). It has been reported that ENCCs produce neurons and glia during their migration. About 10-15% of the ENCC population commences differentiation into neurons at E10.5, and this gradually increases to ~25% by E12.5 (Young et al., 2002). As reported previously, differentiation was already detected at E10.5 (8% in the

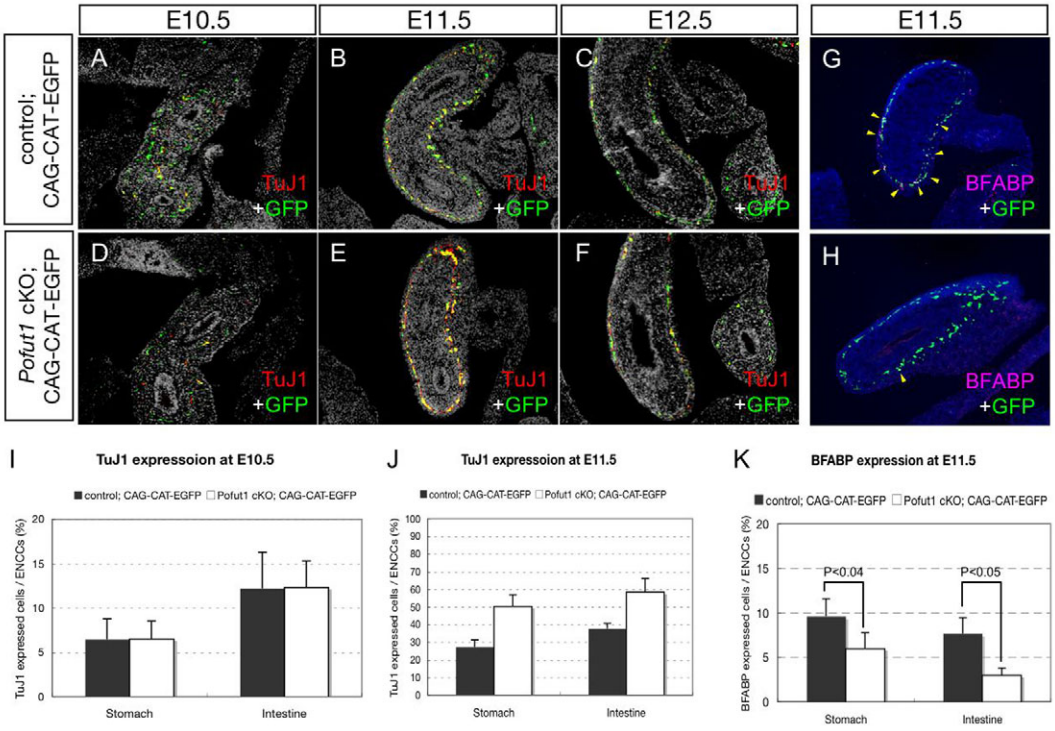

Fig. 4. Neuronal and glial differentiation of ENCCs. Immunohistochemical analysis of control (**A-C**,**G**) and *Pofut1* cKO (**D-F**,**H**) mouse embryos with anti-TuJ1 (red) and anti-GFP (green) antibodies to visualize neuronal cells and ENCCs, respectively, at E10.5-12.5 (A-F), or with anti-BFABP (magenta) and anti-GFP (green) antibodies to visualize glial progenitors and ENCCs, respectively, at E11.5 (G,H). Arrowheads (G,H) indicate BFABP-positive ENCCs. (**I**,**J**) Quantitation of neuronal differentiation at E10.5 (I) and E11.5 (J). Average percentages of TuJ1-positive ENCCs are shown with s.d. (**K**) Quantitation of glial differentiation at E11.5. Average percentages of BFABP-positive ENCCs are shown with s.d.

intestine), and increased to ~37% by E11.5 in our control embryos (Fig. 4A-C,I,J). By contrast, although the proportion of Tuj1-positive cells in the *Pofut1* cKO embryos was not significantly different from that of control embryos at E10.5 (Fig. 4D,I), differentiation was strongly induced thereafter in ~77% of the ENCCs in the *Pofut1* cKO embryos at E12.5 (Fig. 4F). Premature neurogenesis could already be observed at E11.5 (Fig. 4E,J), which is before a reduction in the number of these cells was detectable (Fig. 2C,G).

In addition to neural differentiation, glial differentiation was examined using an anti-BFABP (Fabp7 – Mouse Genome Informatics) antibody (Fig, 4G,H,K), which is a marker for glial progenitors. Glial differentiation is initiated around E11.5 (Young et al., 2003). In *Pofut1* cKO embryos, however, the generation of glial progenitors was reduced at E11.5 (Fig. 4H,K). Therefore, premature neuronal differentiation is likely to be the major reason for the observed reduction in the ENCC number in the *Pofut1* cKO embryos. Hence, we speculated that this premature differentiation might be due to a defect in the maintenance of progenitor cells, which would indicate that Notch signaling is required for the maintenance of ENS progenitors.

Notch signaling is required for the maintenance of Sox10 expression in ENCCs

We next addressed the question of how Notch signaling regulates the pluripotency of ENS progenitors by analyzing the expression of Sox10, which is essential for the maintenance of neural crest progenitors during ENS development (Bondurand et al., 2006; Paratore et al., 2002). The Sox10 expression profile did not differ significantly between control and *Pofut1* cKO embryos at E10.5 (Fig. 5A,B,E,F,I). However, the number of ENCCs expressing Sox10 was significantly reduced, to ~22% of the total population (in the intestine), in *Pofut1* cKO embryos at E11.5 (Fig. 5G,H,J). By contrast, Sox10 expression was observed in 62% of ENCCs (in the intestine) in control embryos (Fig. 5C,D). After E12.5, however, virtually no ENCCs expressed Sox10 in *Pofut1* cKO embryos (data not shown). This suggests that Notch signaling is necessary for the maintenance of Sox10 expression in ENCCs after E11.5.

To investigate the relationship between the decrease in Sox10 expression and the proliferative ability of ENCCs, we examined the proliferation rate of Sox10-positive ENCCs. At E11.5, this did not differ significantly between *Pofut1* cKO (11% in the stomach and the intestine) and control (10% in the stomach, 14% in the intestine) embryos (Fig. 5K). The proliferation rate of Sox10-negative ENCCs, estimated from that of total ENCCs and Sox10-positive ENCCs (Fig. 3Q and Fig. 5J), was ~26% in the stomach and ~30% in the intestine of control embryos, but only ~14% in the stomach and ~16% in the intestine of *Pofut1* cKO embryos. Although these are only estimates of the proliferation rate, the rate for Sox10-negative ENCCs was nonetheless significantly reduced in *Pofut1* cKO embryos. Since Sox10-negative ENCCs may represent cells committed to the neural lineage, these results suggest that proliferation defects are indeed secondary to the premature appearance of cells committed to the neural lineage. Hence, the decreased proliferation and premature neurogenesis observed in *Pofut1* cKO embryos are likely to be a consequence of the downregulation of Sox10 expression, which could then lead to a diminished progenitor population that eventually results in the loss of NCCs after E12.5. However, it was reported recently that Edn/Ednrb signaling is also required for the maintenance of ENS

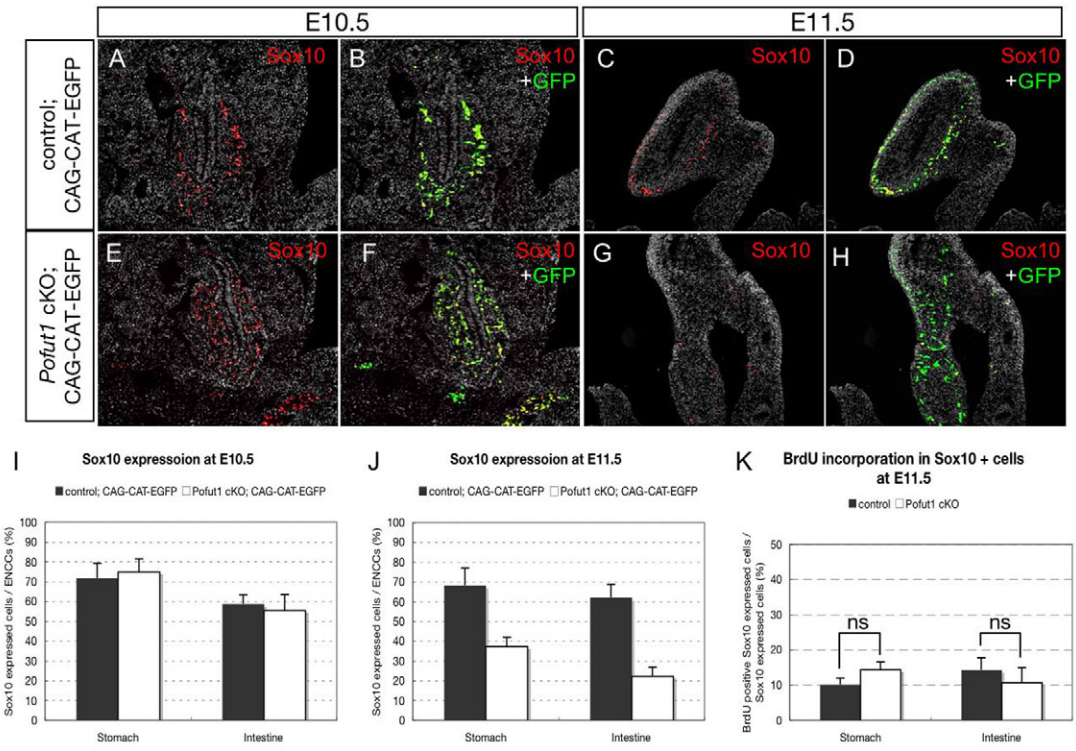

Fig. 5. Downregulation of Sox10 in *Pofut1*-null ENCCs. (**A-H**) Immunohistological detection of Sox10 (red) expression within ENCCs (green) in control (A-D) and *Pofut1* cKO (E-H) mouse embryos at E10.5 (A,B,E,F) and E11.5 (C,D,G,H). (**I**,**J**) Quantitation of Sox10-positive cells at E10.5 (I) and E11.5 (J). Average percentages of Sox10-positive ENCC cells are shown with s.d. (**K**) Quantification of the proliferation rate in Sox10-positive ENCCs at E11.5. Average percentages of BrdU incorporation in proliferating Sox10-positive ENCC cells are shown with s.d. ns, not significant.

progenitors (Bondurand et al., 2006) and that the expression of *Ednrb* in ENCCs is regulated by Sox10 (Zhu et al., 2004). In this context, it is noteworthy that we observed impaired *Ednrb* expression in *Pofut1* cKO embryos (see Fig. S3 in the supplementary material), which is consistent with the downregulation of Sox10 expression in these embryos. Hence, the progenitor maintenance defect in the *Pofut1* cKO embryo might also be due to the loss of Edn/Ednrb signaling.

Expression patterns of Notch receptors and ligands in ENCCs

Since the downregulation of Sox10 expression was observed at E11.5 in *Pofut1* cKO embryos, we speculated that Notch signaling might be activated at around E11.5. However, there have been no previous reports regarding the involvement of Notch ligands or their receptors in ENCC development. We examined the expression of all the known Notch receptors and ligands (*Notch1-4*, *Dll1*, *Dll3*, *Dll4*, *Jag1* and *Jag2*) by in situ hybridization (Fig. 6). The expression of these factors in ENCCs was confirmed by immunohistochemistry with the NCC marker $p75^{NTR}$ (Ngfr – Mouse Genome Informatics) concomitant with in situ hybridization analysis (see Materials and methods). *Notch1*, *Notch4*, *Dll1*, *Dll3*, *Dll4* and *Jag1* were found to be expressed in the gastrointestinal tract. A number of ENCCs expressed *Notch1*, *Dll1* and *Dll3*, but only a few expressed *Notch4*, *Dll4* and *Jag1* (Fig. 6; see Fig. S4 in the supplementary material).

Since ENCCs are known to migrate within the gut mesenchyme as chains of cells (Druckenbrod and Epstein, 2005; Young et al., 2004), we speculated that Notch signaling might be activated through cell-cell contact among NCCs. This idea might be supported by our current finding that both Notch receptors and ligands are expressed in ENCCs. However, some other cell types neighboring the ENCCs were also found to express *Dll4* and *Jag1* (Fig. 6; see Fig. S4 in the supplementary material) and the activation of Notch signaling through neighboring cells might therefore also occur. To investigate ligand redundancy and Notch activation through neighboring cells, we examined Sox10 expression in *Dll1* mutant embryos. *Dll1* mutant embryos did not show clear downregulation of Sox10 expression in post-migratory ENCCs at E11.5 (see Fig. S5 in the supplementary material), indicating that other ligands might also be functional during the maintenance of ENS progenitors.

Regulatory mechanism of Sox10 maintenance

A question that arises from our current data is how Notch signaling regulates Sox10 expression. We speculated that Sox10 might be a direct target of Notch signaling. However, it has been shown that a subset of NCCs expressing Sox10 induces Mash1, which in turn represses Sox10 (Kim et al., 2003). In addition to this negative-feedback mechanism, Mash1 is known to be repressed by Hes1, one of the targets of Notch signaling in the CNS (Chen et al., 1997). These relationships give rise to the possibility that Sox10 expression is maintained by the repression of Mash1 through Notch signaling. This likelihood can be further evaluated by examining Hes1 and Mash1 expression.

If Notch signaling directly activates Sox10 expression, we predicted that Mash1 expression would not be altered or downregulated in *Pofut1* cKO embryos. However, if Notch signaling maintains Sox10 expression by suppressing Mash1 via Hes1, then Hes1 expression would be expected to be downregulated and Mash1 expression to be upregulated in the *Pofut1* cKO mice. We examined these expression patterns in the ENS at E10.5, before

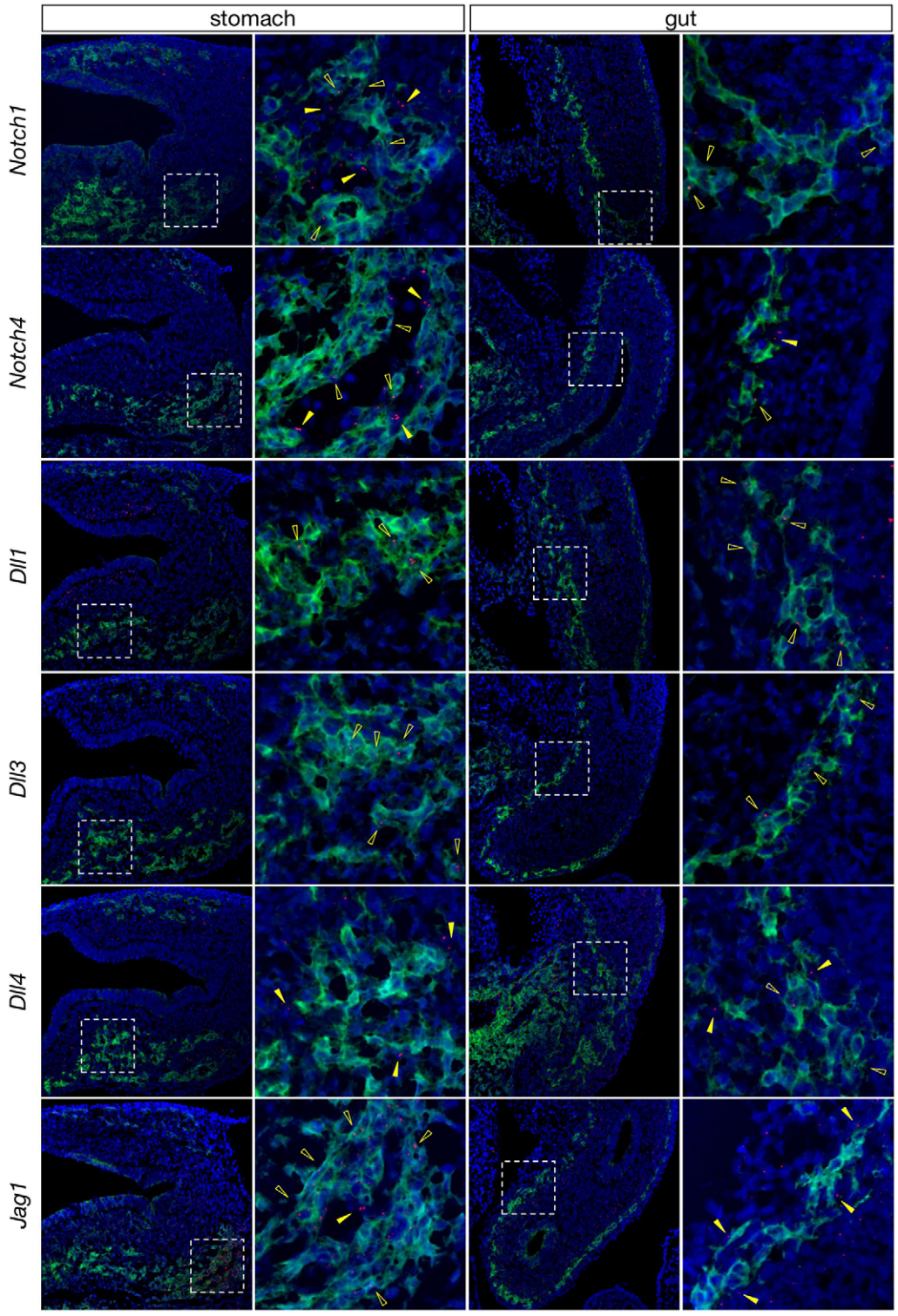

Fig. 6. Expression of Notch signaling genes in the mouse gastrointestinal tract. In situ hybridizations were performed using RNA probes against *Notch1*, *Notch4*, *Dll1*, *Dll3*, *Dll4* and *Jag1* (magenta), together with the immunohistological detection of NCCs using an anti-$p75^{NTR}$ antibody (green) in E11.5 stomach and intestine. Open arrowheads indicate expression in ENCCs, and solid arrowheads indicate expression in neighboring cells. The boxed regions are shown at high magnification in the right-hand panel of each pair.

premature neurogenesis occurs, in *Pofut1* cKO embryos (Fig. 7). In control embryos, Hes1 expression was observed in ~16% of ENCCs in the intestine and also in other non-ENCCs (Fig. 7A-C). By contrast, Hes1 expression in ENCCs was found to be downregulated to ~7% of the total population in the intestine of *Pofut1* cKO embryos (Fig. 7D-F,M). Mash1 expression is restricted in ENCCs and ~48% of ENCCs were indeed found to be Mash1-positive in the intestine of control embryos at this stage (Fig. 7G-I). However, Mash1-positive ENCCs were significantly increased in *Pofut1* cKO embryos (up to ~70% of the intestinal population) (Fig. 7J-L,N), indicating that Notch signaling indirectly regulates Sox10 expression through the suppression of Mash1.

To confirm this scenario, we performed double-immunostaining experiments for Sox10 and Mash1 (Fig. 8) and from their expression patterns the ENCCs were classified into three populations: Sox10 or Mash1 single-positive, and Sox10/Mash1 double-positive. In control embryos, the Sox10/Mash1 double-positive ENCCs (58% of the population in the intestine) were observed from E10.5 and

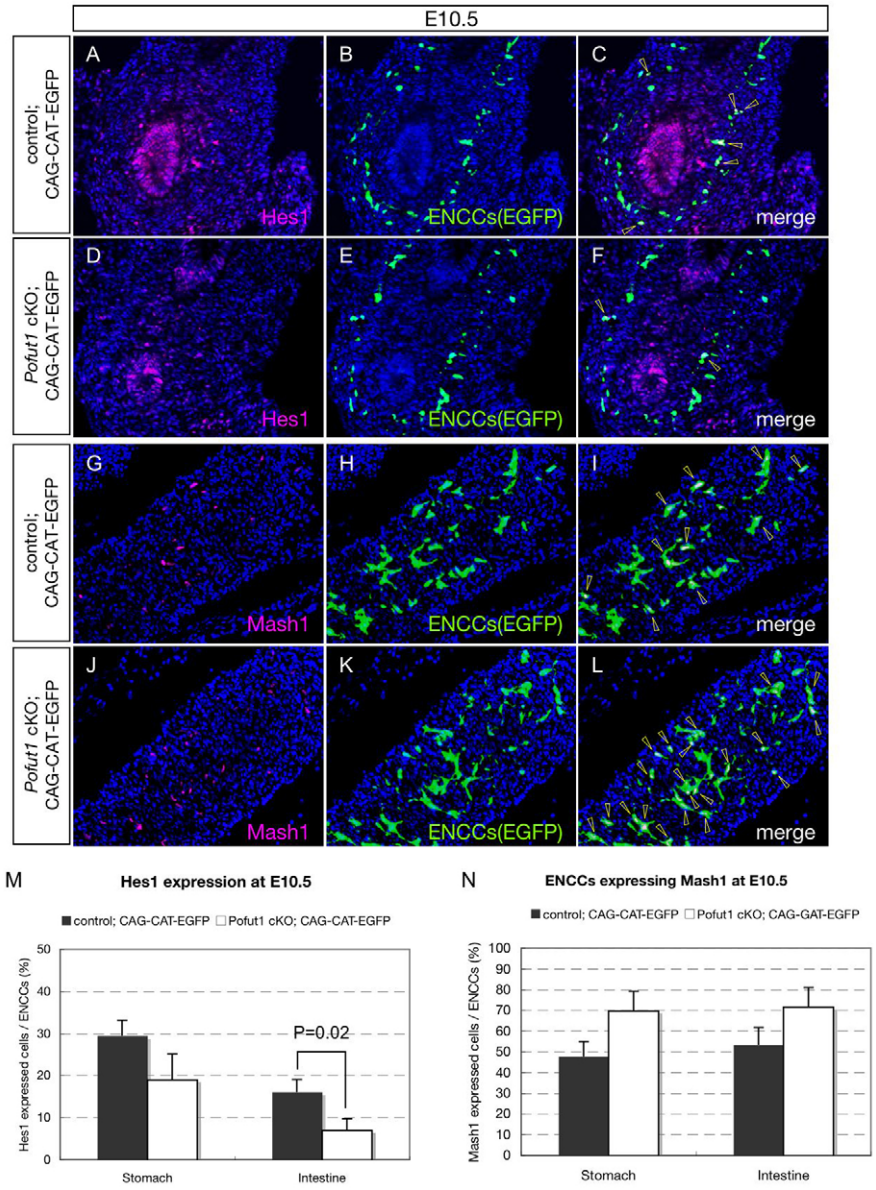

Fig. 7. ENCCs expressing Hes1 are decreased, whereas those expressing Mash1 are increased, in the *Pofut1* cKO embryo. Immunohistological analysis of control (**A-C**,**G-I**) and *Pofut1* cKO (**D-F**,**J-L**) mouse embryos at E10.5 using antibodies against Hes1 (A,C,D,E, magenta), Mash1 (G,I,J,L, magenta) and GFP (B,C,E,F,H,I,K,L, green). Arrowheads indicate Hes1-positive or Mash1-positive ENCCs in control (C,I) and *Pofut1* cKO (F,L) embryos. (**M**,**N**) Quantification of Hes1-positive ENCCs (M) and Mash1-positive ENCCs (N) at E10.5. Average percentages of the total ENCC cell population expressing each gene are shown with s.d.

continually thereafter during embryogenesis (data not shown), but the proportion of Mash1 single-positive cells was low (12% in the intestine) compared with that of Sox10 single-positive cells (30% in the intestine) (Fig. 8C). As expected, however, in *Pofut1* cKO embryos, the abundance of Mash1 single-positive ENCCs was increased (28% in the intestine) and Sox10 single-positive ENCCs were decreased (13% in the intestine) (Fig. 8F,G). The proportion of Sox10/Mash1 double-positive progenitor cells was not significantly different between the control (58% in the intestine) and *Pofut1* cKO (59% in the intestine) embryos (Fig. 8G), indicating that Notch signaling may not participate in the induction of Mash1 expression itself. Hence, Notch signaling appears to regulate the population of Mash1 single-positive ENCCs and be required for the maintenance of enteric neural crest progenitors via the regulation of a Sox10/Mash1 expression balance. Importantly, the balance of transcription factor expression in *Pofut1* cKO embryos was found to have shifted at E10.5, which is prior to the occurrence of the premature differentiation observed only after E11.5. This may indicate that the decrease in the proliferation rate in *Pofut1* cKO embryos is secondary to the premature appearance of ENCCs that are committed to the neuronal lineage. In other words, altering the balance of expressed transcription factors at E10.5, which results in the upregulation of Mash1, may cause premature differentiation and a decrease in proliferation at E11.5 and, eventually, a reduction in the number of ENCCs at E12.5.

DISCUSSION

We performed a comprehensive series of expression analyses in the *Pofut1* cKO mouse in combination with a lineage tracer and demonstrate that Notch signaling plays a crucial role during ENS development in vivo. In general, this strategy can address the function of Notch signaling in all neural crest derivatives. Mice lacking Pofut1 in NCCs had a slightly smaller body size than their control littermates. This was probably caused by a defect in a subset of the neural crest derivatives in which Notch signaling plays some role. However, the *Pofut1* cKO embryos did not exhibit obvious abnormalities in regions such as the craniofacial structures and heart arteries. Hence, the smaller body size might be a secondary effect of Notch signaling impairment in neural crest derivatives that were not included in our current analysis.

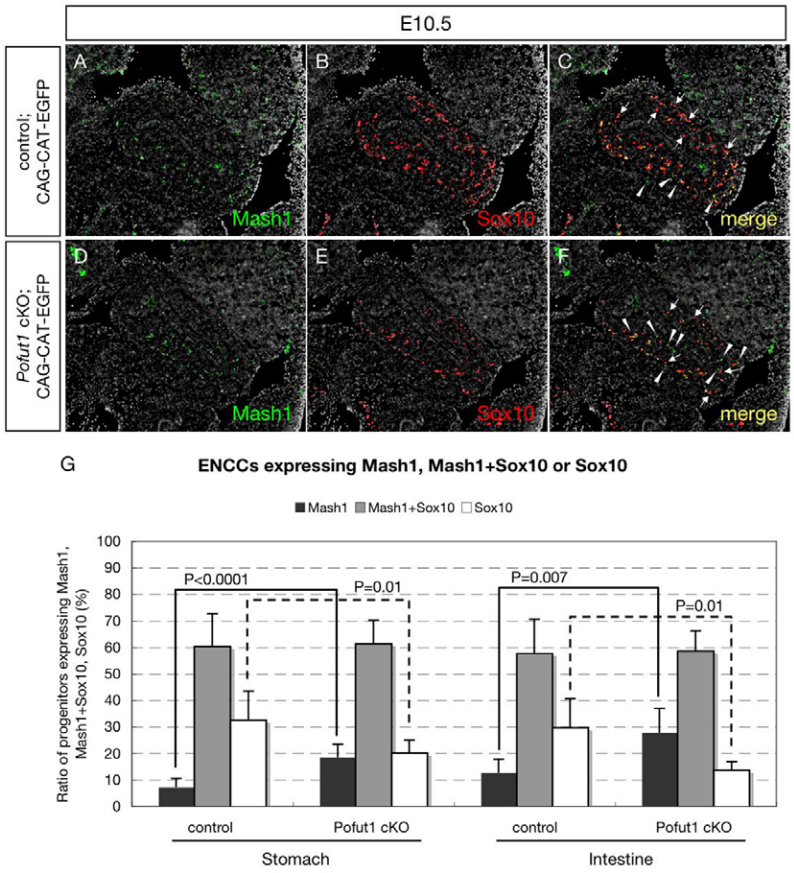

Fig. 8. Comparative analysis of ENCCs expressing Mash1 and Sox10. Immunohistological analysis of control (**A-C**) and *Pofut1* cKO (**D-F**) mouse embryos at E10.5 using antibodies against Mash1 (A,C,D,F, green) and Sox10 (B,C,E,F, red). Arrowheads indicate Mash1 single-positive ENCCs, and arrows indicate Sox10 single-positive ENCCs. (**G**) Statistical analyses of Mash1-single positive, Sox10-single positive or Mash1/Sox10 double-positive cells at E10.5. Average percentages of the ENCC progenitor population expressing Mash1 or Sox10 are shown with s.d.

Phenotypic variation in mutants showing defective ENS development

Mice lacking Pofut1 in their NCCs showed a reduction in ENCCs at E12.5, although these cells retained their capacity to contribute to development throughout the gastrointestinal tract. This phenotype appears to be different from the defects observed in mouse models of human congenital megacolon (Hirschsprung disease), including *Sox10*, *Edn3*, *Ednr*, *Gdnf*, *Ret* and *Gfra1* knockout mice (Enomoto et al., 1998; Herbarth et al., 1998; Schuchardt et al., 1994; Southard-Smith et al., 1998; Stanchina et al., 2006). Each of these mutant mice shows a complete loss of enteric neurons in the gut region that is distal to the stomach. In addition, some of them, including *Gdnf*, *Ret* and *Gfra1* knockout mice, show milk in their esophagus but die within 1 day of birth.

It has been reported that *Mash1*-null mutants also die within 1 day of birth but without milk in their stomachs (Guillemot et al., 1993). Interestingly, *Mash1* mutants lack neurons in the esophagus but possess these cells in the stomach and intestine. There might be a correlation, therefore, between milk in the stomach and the presence of neurons in the esophagus. However, *Pofut1* cKO embryos, in which neurons are present in the esophagus, but are reduced in number in the stomach and intestine, had no milk in their stomach. The reason for this is currently unknown.

Relationship between Notch signaling and glial development in the ENS

Recently, experiments that are similar to those of our current study were conducted using *RBP-Jk*^{flox} and *Wnt1-Cre* mice. The authors of this earlier study concluded that Notch signaling promotes gliogenesis in the ENS, based upon their finding that neurogenesis was less affected, but gliogenesis severely affected, by the gene knockout (Taylor et al., 2007). This apparent inconsistency with our current data might be due to differences in the methods used and in the time windows analyzed, as we obtained results similar to those reported here when we performed comparable experiments using *RBP-Jk*^{flox} and *Wnt1-Cre* mice (data not shown). We also employed a linage tracer to analyze specific gene expression patterns in ENCCs, and this might have affected the interpretation of results even though similar probes were used. In our lineage analysis, we observed a clear reduction in the number of ENCCs at E12.5 in *Pofut1* cKO embryos (Fig. 2). However, Taylor et al. (Taylor et al., 2007) counted differentiated ENCCs in *RBP-Jk* cKO embryos at E10.5, E14.5 and E18.5, by which time decreased proliferation and any premature neurogenesis occurring at E11.5 could have been missed. Since Sox10 is expressed not only in the ENS progenitors, but also in the glial cell lineage, the downregulation of Sox10 expression observed in the *Pofut1* cKO embryo at E11.5 must lead to the loss of glial progenitors thereafter. Hence, we support the notion that both the maintenance of ENS progenitors and the formation of glial progenitors are accounted for by the maintenance of Sox10 expression. Taken together, we conclude from the available data, including our current results, that Notch signaling is required for the maintenance of ENS progenitors rather than for the promotion of glial cell differentiation.

A model for the mechanism underlying the maintenance of ENS progenitors

The ENCCs in *Pofut1* cKO embryos exhibited premature differentiation without increased apoptosis and with a slight decrease in proliferation during ENS development. These results

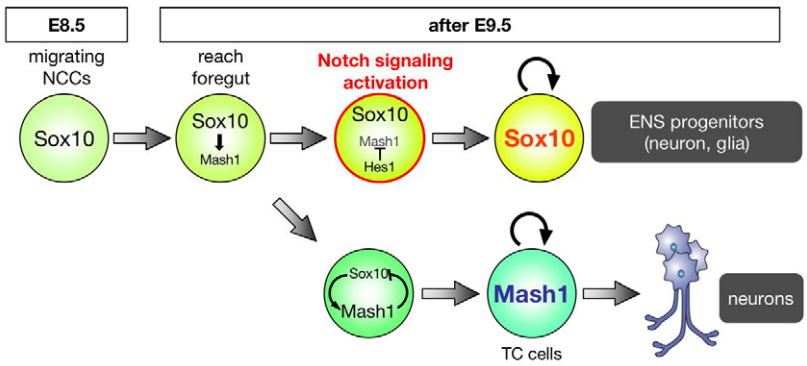

Fig. 9. A hypothetical model for the function of Notch signaling during mouse ENS development. The NCCs begin to express Sox10 when they detach from the neural tube at around E8.5. After arriving at the foregut (E9.5), a subset of the NCCs begins to express Mash1. In turn, Mash1 suppresses Sox10 expression, and Mash1-positive cells then eventually produce neurons. The remaining NCCs continue to express Sox10 to maintain the pool of ENS progenitors. Notch signaling might be required for the continuous expression of Sox10 by suppressing Mash1. TC cells, transient catecholaminergic cells (see Discussion).

suggest that Notch signaling is necessary for the maintenance of ENS progenitors. Since this finding is consistent with a role for Notch signaling in the CNS, it may well reflect a common role for Notch during neurogenesis.

Previous studies have shown that the lack of some progenitors or neurons is simply explained by the lack of particular transcription factors, which include the maintenance of NCC progenitors by Sox10 and the induction of some of the enteric neuron lineages by Mash1. However, our current study demonstrates for the first time that progenitor maintenance and the subsequent differentiation of ENCCs are regulated by the balance of Sox10 and Mash1 expression, which might enable a continuous derivation of mature neurons while maintaining a constant progenitor population. We also demonstrate herein that Notch signaling plays a crucial role in maintaining this balance by suppressing Mash1 expression (Fig. 9).

Further interesting aspects of Mash1-expressing cells have emerged from previous studies. In the developing gut, catecholaminergic (TC) cells have been transiently detected and are thought to be the precursors of a subset of enteric neurons as they show proliferative ability (Baetge et al., 1990). Both the selective elimination of TC cells and a *Mash1*-null mutation prevent the generation of a subset of enteric neurons. However, the remaining ENCCs can still produce another class of enteric neurons (Blaugrund et al., 1996). From this observation, it has been proposed that the ENS comprises two progenitor populations. One comprises the TC cells that give rise to early-born enteric neurons via a Mash1-dependent pathway, and the other contains a population of non-TC cells that generate late-born enteric neurons via a Mash1-independent pathway (Blaugrund et al., 1996). Since the ENCCs that express Sox10 are a cell population that is distinct from TC cells (Young et al., 2002), this suggests that non-TC cells can be formed by a Sox10-dependent pathway. In addition, Sox10/Mash1 double-positive ENCCs were observed in control embryos throughout embryogenesis (Fig. 8 and data not shown). Hence, it is intuitive that the expression of these transcription factors must be balanced in ENCCs, even though one of the functions of Mash1 is to suppress Sox10 expression. We speculate that Notch signaling is involved in regulating the balance between Sox10 and Mash1 expression, which may in turn generate two progenitor populations, one Mash1-dependent and the other Sox10-dependent (Fig. 9).

Although many genes involved in ENS development have been identified in recent years, their regulatory mechanisms and the relationships between the different gene functions remain poorly understood. Our current study provides new insights into these pathways by demonstrating that the cellular properties of ENCCs are regulated by a cell-cell communication mechanism that is mediated by Notch signaling. Further analyses of the molecular mechanisms underlying this cell-cell communication might provide a more precise understanding of the developmental processes within the ENS that might be applied in future therapeutic strategies against Hirschsprung disease.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/21/3555/DC1

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