Development 133, 4507-4516 (2006) doi:10.1242/dev.02616

# Targeted mutation of serine 697 in the *Ret* tyrosine kinase causes migration defect of enteric neural crest cells

**Naoya Asai[1,2], Toshifumi Fukuda[3], Zaiqi Wu[2], Atsushi Enomoto[1], Vassilis Pachnis[4], Masahide Takahashi[1,*,†] and Frank Costantini[2,*]**

The RET receptor tyrosine kinase plays a critical role in the development of the enteric nervous system (ENS) and the kidney. Upon glial-cell-line-derived neurotrophic factor (GDNF) stimulation, RET can activate a variety of intracellular signals, including the Ras/mitogen-activated protein kinase, phosphatidylinositol 3-kinase (PI3K)/AKT, and RAC1/JUN $NH_2$-terminal kinase (JNK) pathways. We recently demonstrated that the RAC1/JNK pathway is regulated by serine phosphorylation at the juxtamembrane region of RET in a cAMP-dependent manner. To determine the importance of cAMP-dependent modification of the RET signal in vivo, we generated mutant mice in which serine residue 697, a putative protein kinase A (PKA) phosphorylation site, was replaced with alanine (designated S697A mice). Homozygous S697A mutant mice lacked the ENS in the distal colon, resulting from a migration defect of enteric neural crest cells (ENCCs). In vitro organ culture showed an impaired chemoattractant response of the mutant ENCCs to GDNF. JNK activation by GDNF but not ERK, AKT and SRC activation was markedly reduced in neurons derived from the mutant mice. The JNK inhibitor SP600125 and the PKA inhibitor KT5720 suppressed migration of the ENCCs in cultured guts from wild-type mice to comparable degrees. Thus, these findings indicated that cAMP-dependent modification of RET function regulates the JNK signaling responsible for proper migration of the ENCCs in the developing gut.

**KEY WORDS: RET tyrosine kinase, GDNF, Enteric nervous system, Protein kinase A, JNK, Mouse**

## INTRODUCTION

The *Ret* proto-oncogene encodes a receptor tyrosine kinase that is essential for the development of the enteric nervous system (ENS) and the kidney. In mice, targeted disruption of *Ret* causes intestinal aganglionosis and renal agenesis or severe hypoplasia (Enomoto et al., 2001; Schuchardt et al., 1994). It has also been demonstrated that RET signaling is required for the proper development of parasympathetic neurons and motoneurons as well as spermatogonia (Airaksinen and Saarma, 2002; Enomoto et al., 2000; Meng et al., 2000). In humans, germline mutations of *Ret* lead to the development of Hirschsprung's disease, which is characterized by the absence of enteric ganglia from the distal gastrointestinal tract (Edery et al., 1994; Romeo et al., 1994).

RET is activated by the glial-cell-line-derived neurotrophic factor (GDNF) family of ligands, which include GDNF, neurturin, artemin and persephin (Airaksinen and Saarma, 2002). Unlike other receptor tyrosine kinases, glycosyl phosphatidylinositol-linked cell-surface proteins called GDNF family receptor α1-4 (GFRα1-4) are required as ligand-binding components for RET activation (Airaksinen and Saarma, 2002; Jing et al., 1996; Klein et al., 1997; Treanor et al., 1996). *Gdnf*- or *Gfrα1*-deficient mice also showed phenotypes similar to *Ret*-deficient mice, confirming that complex formation of these three molecules is crucial for the development of the ENS and the kidney (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). In the developing ENS, RET is expressed in migrating enteric neural crest cells (ENCCs) whereas GDNF is expressed in the mesenchyme of the gut in a spatially and temporally regulated manner (Natarajan et al., 2002). In the developing kidney, RET and GDNF are expressed in the branching ureteric buds and metanephric mesenchyme, respectively (Hellmich et al., 1996; Pachnis et al., 1993). In addition, it has been reported that RET signaling activated by neurturin, artemin or persephin plays an important role in survival, proliferation and/or differentiation of various types of central and/or peripheral neurons (Airaksinen and Saarma, 2002).

RET can activate a variety of intracellular signaling pathways, including Ras/mitogen-activated protein kinase (MAPK), PI3K/AKT, RAC1/Jun $NH_2$-terminal kinase (JNK), p38MAPK and phospholipase Cγ pathways (Ichihara et al., 2004; Kodama et al., 2005). RET can also activate RHO family GTPases, including RHO, RAC and CDC42, which are involved in reorganization of the actin cytoskeleton necessary for cell motility (Barone et al., 2001; Chiariello et al., 1998; Maeda et al., 2004; Murakami et al., 1999; van Weering and Bos, 1997). Moreover, Encinas et al. (Encinas et al., 2001) reported that SRC kinase activity is necessary to elicit optimal GDNF-mediated signaling, neurite outgrowth and survival.

Recently, we demonstrated that phosphorylation of the serine residue at codon 696 (S696) of RET via a cAMP-dependent mechanism is required for RAC1-GEF activation and lamellipodia formation in the human SK-N-MC neuroectodermal tumor cell line transfected with the human *RET* gene (designated MC(RET) cells) (Fukuda et al., 2002; Fukuda et al., 2005). When S696 was replaced with alanine (S696A), GDNF-mediated activation of RAC1-GEF and lamellipodia formation were abolished. We found that S696 represents a protein kinase A (PKA) phosphorylation site and that treatment with the PKA inhibitor impaired these GDNF-dependent activities. Phosphorylation of tyrosine 687 (Y687) and S696 at the juxtamembrane region appeared to induce opposite effects on lamellipodia formation, which suggested the possibility that S696

[1]Department of Pathology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. [2]Department of Genetics and Development, Columbia University, 701 West 168th Street, New York, NY 10032, USA. [3]Laboratory of Molecular Biochemistry, School of Life Science, Tokyo University of Pharmacy and Life Science, Tokyo 192-0392, Japan. [4]Division of Molecular Neurobiology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

*These authors contributed equally to this work
†Author for correspondence (e-mail: mtakaha@med.nagoya-u.ac.jp)

**Accepted 6 September 2006**

phosphorylation suppresses the signal arising from phosphorylated Y687 residue (Fukuda et al., 2002). In addition, we found that the RAC1/JNK signaling pathway was specifically impaired in S696A cells and that GDNF stimulation caused G2/M cell-cycle delay in MC(RET) cells but not in S696A cells (Fukuda et al., 2005).

To investigate the in vivo role of RET signaling regulated by PKA, we used targeted mutagenesis in embryonic stem (ES) cells to replace the putative serine phosphorylation site of codon 697 in mouse RET (corresponding to serine 696 in human RET), with alanine (RET S697A mutation). RET S697A mutant mice showed aganglionosis in the distal colon, resulting from migration defects of ENCCs in the developing gut. Consistent with our previous results, GDNF-mediated JNK activation was specifically impaired in neurons derived from the mutant mice. These findings reveal that PKA-dependent RET phosphorylation at S697 regulates the JNK signaling pathway and controls the migration of ENCCs in the developing gut.

## MATERIALS AND METHODS

### Generation of RET S697A mutant mice

A 7.0 kb *Bam*HI/*Hin*dIII fragment encompassing exon 9 to exon 12 of the mouse *Ret* gene was used to construct the targeting vector (Fig. 1C). To introduce the S697A mutation in exon 11, PCR mutagenesis was used. A loxP-flanked Pgk/*neo* gene was inserted at the *Apa*I site in intron 11. The linearized targeting vector was electroporated into W9.5 ES cells (a gift of Dr Colin Stewart), and homologous recombinants were selected by growth in G418. Targeted ES cell clones were identified by Southern blot analysis using an external probe made by PCR with the forward primer (5′-CCAAGCTTCAGTACACGGTGGT-3′) and the reverse primer (5′-AGGCCACATACACCTAAGAC-3′) (Fig. 1C,D). The genotype of each mutant mouse was determined using PCR with the forward primer (p1; 5′-ATTCCACGGAGAACCAGG-3′) and the reverse primer (p2; 5′-CTAACACAGGTCTCTTCC-3′) (Fig. 1C,E).

### Antibodies

Anti-RET and anti-phospho-RET(pS697) antibodies were developed as described previously (Fukuda et al., 2002). Anti-PGP9.5 antibody was purchased from UltraClone Limited. Anti-ERK, anti-phospho-ERK, Anti-AKT, anti-phospho-AKT, anti-p38MAPK, anti-phospho-p38MAPK, anti-JNK and anti-phospho-JNK antibodies were purchased from Cell Signaling Technology. Anti-SRC antibody (GD11) and anti-phospho-SRC antibody were purchased from Upstate Cell Signaling Solution and Biosource, respectively. Anti-rabbit Alexa Fluor was purchased from Molecular Probes. Anti-β-actin antibody was purchased from Sigma-Aldrich.

### Explant cultures

For the chemoattractant assay, E11.5 or E12.5 gut segments prepared from midgut were cut and transferred onto the surface of collagen gels (KOKENCELLGEN, Koken) containing 10% fetal bovine serum (FBS). GDNF-soaked agarose beads (Cibacron, Sigma; 80-110 μm diameter) were placed 500-800 μm from gut segments and cultured for 4 days (Young et al., 2001). For in vitro organ culture, embryonic guts were dissected out at E11.5, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. Individual guts were cultured in 60 μl hanging drops and fed every day. To inhibit a specific signaling pathway, 30 μM of MEK1 inhibitor (PD98059), 20 μM of PI3K inhibitor (LY294002), 10 μM of SRC kinase inhibitor (SU6656), 10 μM of JNK inhibitor (SP600125) or 1 μM of PKA inhibitor (KT5720) was added to the medium.

### Primary culture of ENCCs

Primary culture of ENCCs has been described previously (Barlow et al., 2003). Briefly, E11.5 guts were dissected and incubated with Collagenase/Dispase (0.5 mg/ml). Tissues were dissociated into single cells by repeated pipetting and cultured in F12/DMEM supplemented with 10% FBS. GDNF was added at a concentration of 50 ng/ml. After 3 days, cultured cells were immunostained with anti-PGP9.5 antibody.

### Primary culture of dorsal root ganglia

Primary culture of neurons from dorsal root ganglia (DRGs) has been described previously (Jijiwa et al., 2004). Briefly, DRGs were obtained from wild-type and mutant mice at postnatal day 7 and digested with 0.15% collagenase and 0.05% trypsin-EDTA. Isolated cells were cultured in F12/DMEM supplemented with 10% FBS for 3 days. After 2 hours of serum starvation, cultured cells were treated with GDNF (100 ng/ml) for 20 minutes, lysed in sodium dodecyl sulfate sample buffer, and subjected to western blot analysis.

### Histology and immunostaining

In explant gut cultures migrated ENCCs, which were differentiated into neuronal cells, were visualized with anti-PGP9.5 immunostaining as described previously (Young et al., 2001). Explants were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). After washing with PBT (PBS+0.1% Triton X-100) three times, they were incubated for 3 hours with blocking solution (PBS+2% BSA) and incubated with anti-PGP9.5 antibody (1:1000 dilution) at 4°C overnight. Then explants were washed with PBT several times, incubated with anti-rabbit Alexa Flour (1:1000 dilution) at 4°C overnight and analyzed in an Olympus stereomicroscope. To quantify the chemoattractant response to GDNF, PGP9.5-positive areas were subdivided into quadrants, and measured using image analysis software (WinROOF).

Whole-mount acetylcholinesterase (ACHE) histochemistry and whole-mount in situ hybridization were carried out as previously described (Enomoto et al., 1998; Natarajan et al., 2002). ENS cells in the myenteric plexus were stained with cuprolinic blue, and positive cells were counted under a microscope, and calculated as number/mm$^2$. Cells were counted in ten randomly selected fields of each region of the gut from four mice.

## RESULTS

### Introduction of S697A mutation into the mouse *Ret* gene

The mutation S697A was introduced into the mouse *Ret* gene by targeted mutagenesis in W9.5 ES cells (Fig. 1A-C). The mutation in ES cells was confirmed by sequencing of an amplified PCR product containing the mutation site (Fig. 1B). To permit subsequent removal, a *neo* gene was flanked with loxP sites and placed in the intron 11 (Fig. 1C). Two independently targeted ES cell clones were injected into C57BL/6J blastocysts, and both transmitted the RET S697A mutation through the germline (RET$^{S697A\text{-}neo}$). To remove the *neo* gene, RET$^{S697A\text{-}neo}$ mice were crossed with β-actin promoter/Cre transgenic mice (Lewandoski and Martin, 1997), and excision of the *neo* gene was confirmed by Southern blotting (Fig. 1D). Heterozygous RET$^{S697A}$ mice (S697A mice) were intercrossed to generate homozygous S697A mice (Fig. 1E). Because a 70 bp insertion containing a single loxP site and a polylinker sequence was still left in intron 11 in the S697A mice, which may affect the expression of RET, the protein levels of RET in embryos and newborn brain stems and stomachs of wild-type and mutant mice were examined by western blotting. As shown in Fig. 1F, RET proteins were expressed at comparable levels in wild-type, heterozygous and homozygous S697A mice. To investigate the phosphorylation status of Serine 697, DRG neurons were isolated and cultured from wild-type and mutant mice, and analyzed by western blotting with anti-phospho-RET(S697). S697 phosphorylation was clearly observed in wild-type mice but not in mutant mice (Fig. 1G).

### RET S697A mutation affects the development of the ENS in the distal colon

As RET signaling plays a critical role in ENS development, we first analyzed the myenteric plexus of guts in mutant newborn mice by ACHE histochemistry. While heterozygous S697A mice showed an

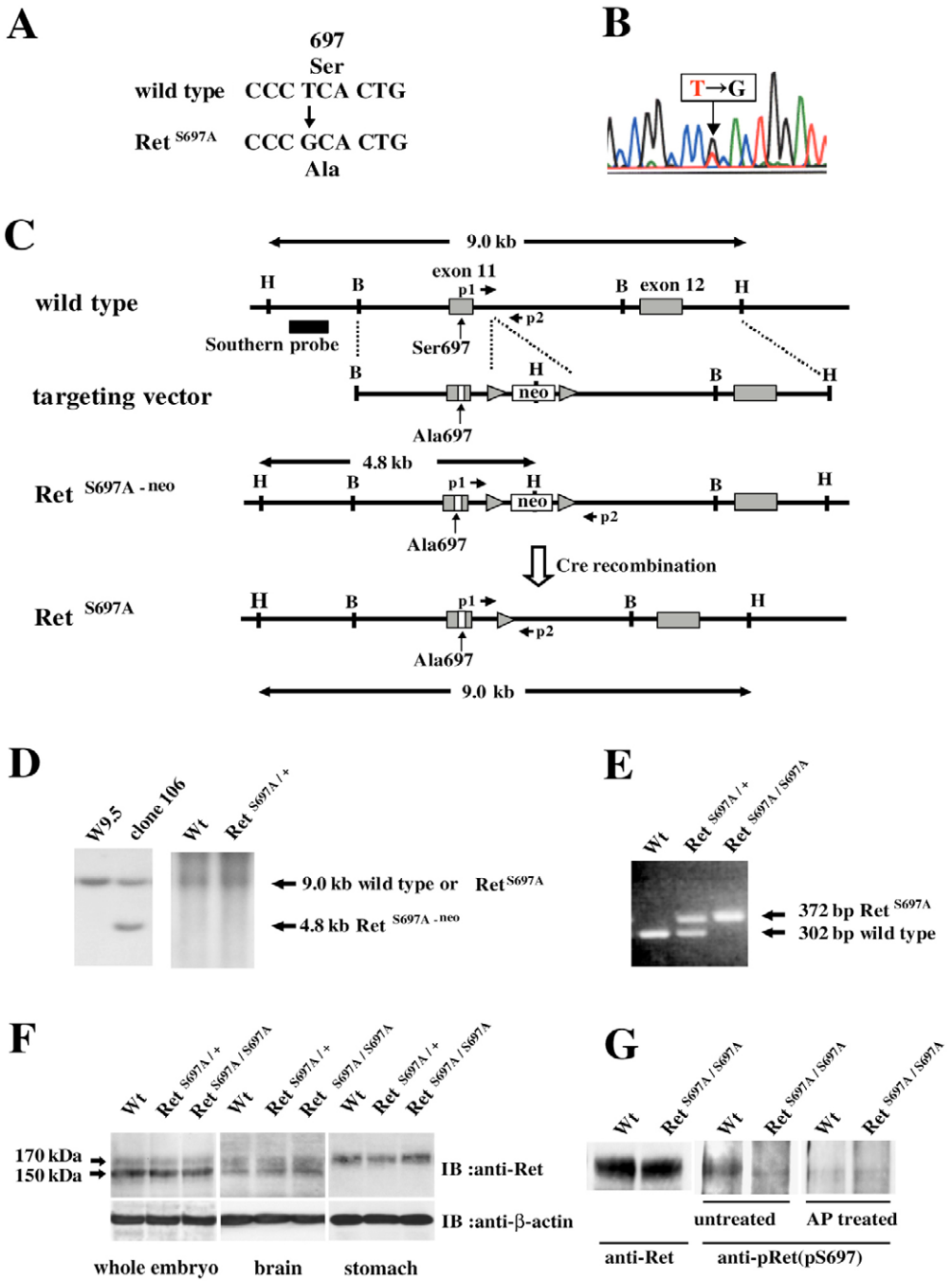


**Fig. 1. Generation of RET S697A mutant mice.** (**A**) The S697A mutation introduced in mouse RET exon 11. (**B**) Sequencing of amplified PCR product containing the S697A mutation site. Template DNA was prepared from clone 106 shown in (D). (**C**) Schematic representation of the targeting strategy. The S697A mutation was introduced in RET exon 11 and the loxP flanked *neo* gene in intron 11 in the targeting vector. After homologous recombination in ES cells, RET$^{S697A\text{-}neo}$ mutant mice were generated. The *neo* gene was removed to generate RET$^{S697A}$ mutant mice by crossing with β-actin promoter/Cre transgenic mice. P1 and P2 indicate the forward and reverse primers for genotyping. H, *Hin*dIII; B, *Bam*HI. (**D**) Southern blot analysis for RET$^{S697A\text{-}neo}$ and RET$^{S697A}$ mutations. Genomic DNA was prepared from control W9.5 ES cells (W9.5), homologous recombinant clone (clone 106), and tails of wild-type (Wt) and heterozygous RET S697A mutant mice (RET$^{S697A/+}$). DNA was digested with *Hin*dIII, and analyzed with the indicated 5′ external probe shown in (C). Wild-type and RET$^{S697A\text{-}neo}$ mutant alleles generate 9.0 kb and 4.8 kb bands, respectively. Loss of 4.8 kb band in DNA from a RET$^{S697A/+}$ mutant mouse indicates successful removal of the *neo* gene. (**E**) Genotyping of mutant mice. P1 and P2 primers shown in (B) were used for genotyping by PCR. As the S697A mutant allele contains a 70 bp insertion, wild-type and mutant alleles give 302 bp and 372 bp bands, respectively. (**F**) RET protein expression in mutant mice. Lysates prepared from whole embryos at E11.5, newborn brain stems and stomachs were subjected to western blotting with anti-RET antibody. The 170 kDa and 150 kDa RET proteins are indicated. β-Actin expression was investigated as a loading control. (**G**) Phosphorylation of Serine 697 in DRG neurons. Lysates were prepared from cultured DRG neurons isolated from wild-type and mutant mice, and subjected to western blotting with anti-RET and anti-phospho-RET(S697) antibodies (left and middle panels). The membrane was treated with alkaline phosphatase (AP) before incubation with anti-phospho-RET(S697) antibody to confirm that the detected band represents the phosphorylated protein (right panel).

apparently normal myenteric plexus throughout the gut (data not shown), homozygous S697A mice displayed a severe reduction or absence of the myenteric plexus in the distal portion of the colon (Fig. 2). In homozygous mutant mice, enteric ganglia were absent in the terminal one-third of the colon (Fig. 2H, compare with D), and significantly reduced in number in the middle one-third (Fig. 2G, compare with 2C). In contrast, ENS development appeared normal in the proximal one-third of the colon (Fig. 2F, compare with 2B). The degree of aganglionosis or hypoganglionosis in the colon was almost identical between male and female mice and between the established two independent mutant mouse lines (data not shown). In the stomach and the small intestine, the density and distribution of the enteric ganglia were indistinguishable between wild-type and homozygous S697A mice (data not shown).

To investigate whether the ENS in the proximal gut is normal in mutant mice, we performed a quantitative analysis of the neuron number in the myenteric plexus using cuprolinic blue staining. The number of ENS cells in the myenteric plexus showed no significant difference between wild-type and mutant mice in the whole region of the small intestine (Fig. 3A) and the proximal portion of the colon (Fig. 3B). In contrast, marked reduction of enteric neurons was observed in the middle and distal portions of the colon in the homozygous S697A mice (Fig. 3B). In addition, ACHE staining showed that fiber density and number and size of the myenteric neural plexus were comparable between wild-type and mutant mice in the small intestine (data not shown). These results demonstrate that reduction of ENS cells occurs in the middle and distal colon of the S697A mutant mice.

## S697A mutation impairs migration of ENCCs in the developing gut

To elucidate why aganglionosis occurred in the distal colon of homozygous S697A mice, we analyzed the ENS development at embryonic stages E11.5-14.5, during which ENCCs migrate within the gut towards the distal part of the hindgut (Young and Newgreen, 2001). At E11.5, RET-positive ENCCs had migrated beyond the cecum in wild-type mice, while in the homozygous S697A mice they were migrating in the small intestine but had not yet arrived at the cecum (Fig. 4A,A′,D,D′). At E12.5, ENCCs arrived at the cecum in the mutant mice (Fig. 4E,E′). At E14.5, wild-type ENCCs reached the terminus of the hindgut, while mutant ENCCs were still migrating in the middle portion of the hindgut (Fig. 4C,C′,F,F′), where marked reduction of enteric neurons was observed in newborn mutant mice. Thus, aganglionosis in the distal colon is likely to be due to the impaired migration of the mutant ENCCs.

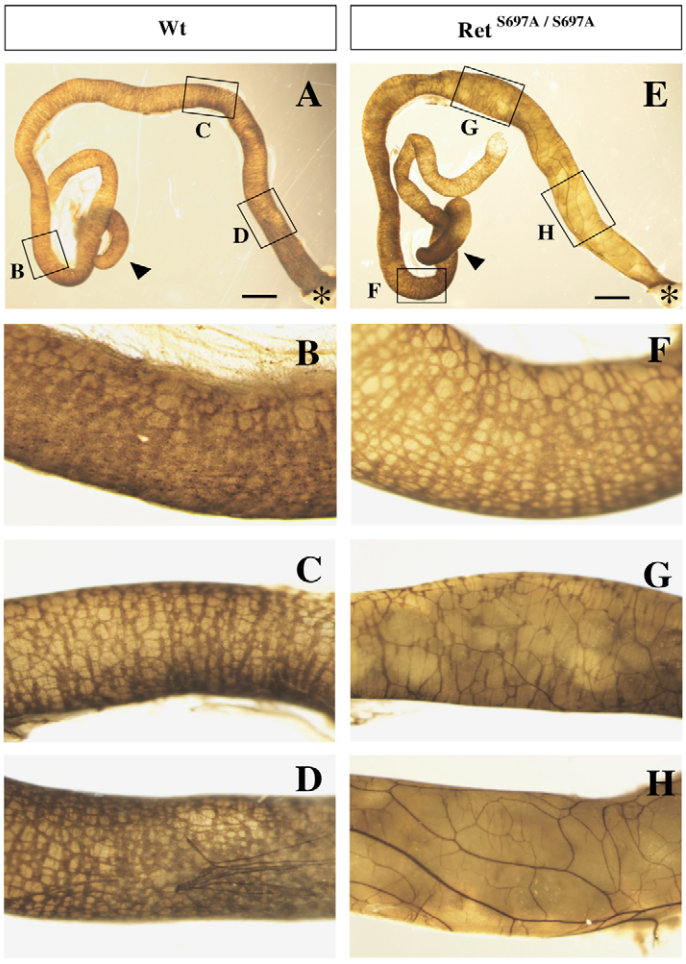


**Fig. 2. Aganglionosis in the distal colon of RET S697A mice.** Whole-mount AchE histochemistry of the distal intestine of wild-type (**A-D**) and homozygous S697A mutant newborns (**E-H**) at P3. Lower magnification views (A,E) and higher magnification views in the proximal colon (B,F), the middle colon (C,G) and the distal colon (D,H) are shown. Homozygous mutant mice exhibited marked reduction of enteric ganglia in the middle colon (G) and their absence in the distal colon (H). Arrowheads, cecum; asterisks, terminus of the colon; bars, 1 mm.

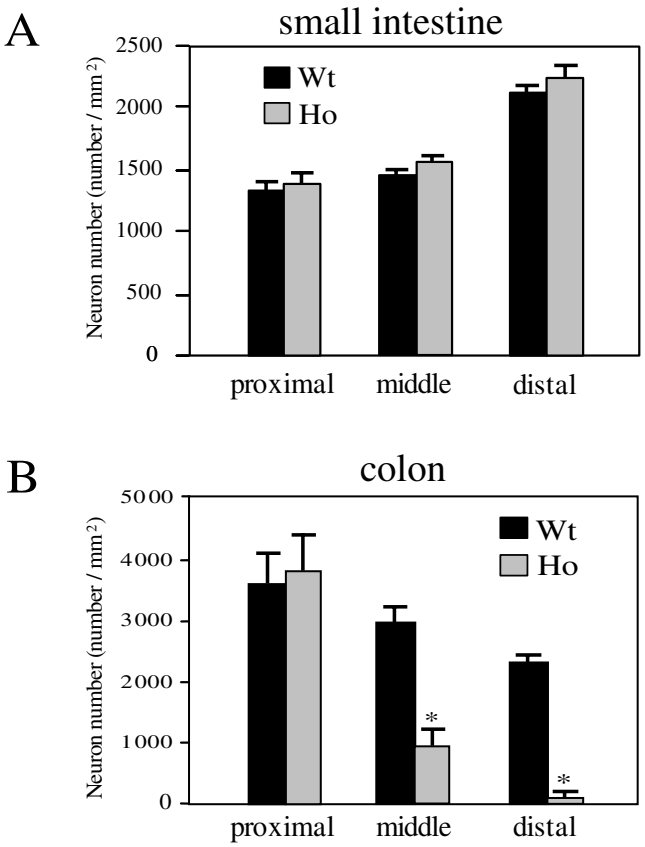


**Fig. 3. Numbers of ENS cells in the gut of RET S697A mutant mice.** ENS cells were stained with cuprolinic blue and cell numbers were counted at each region of the small intestine (**A**) and the colon (**B**). The mean was calculated from ten views of each section of the intestine from four mice at P12. *$P$<0.01 compared with wild-type mice. Ho, homozygotes.

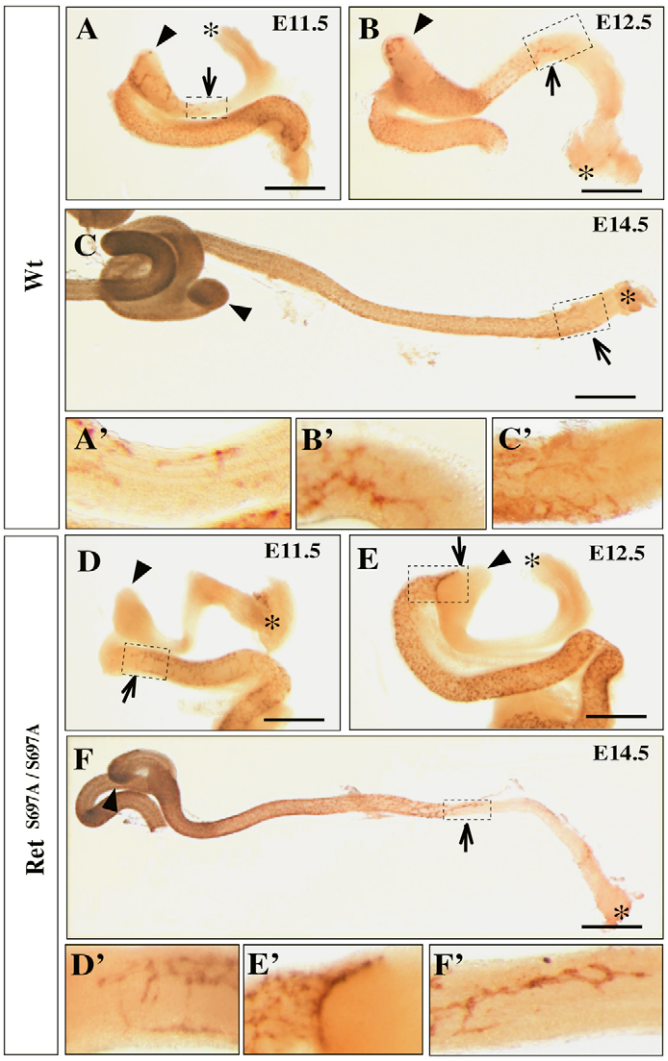


**Fig. 4. Impaired migration of the ENCCs in RET S697A mutant mice.** Whole-mount in situ hybridization with riboprobe specific for RET on the developing guts in wild-type (**A-C**) and homozygous RET S697A mutant mice (**D-F**) at E11.5 (A,D), E12.5 (B,E) and E14.5 (C,F). (**A′-C′**,**D′-F′**) show high-power views of the front of the migrating RET positive ENCCs in A-F, respectively. Arrows, the front of the migrating ENCCs; arrowheads, cecum; asterisks, terminus of the hindgut. Scale bars: 500 μm.

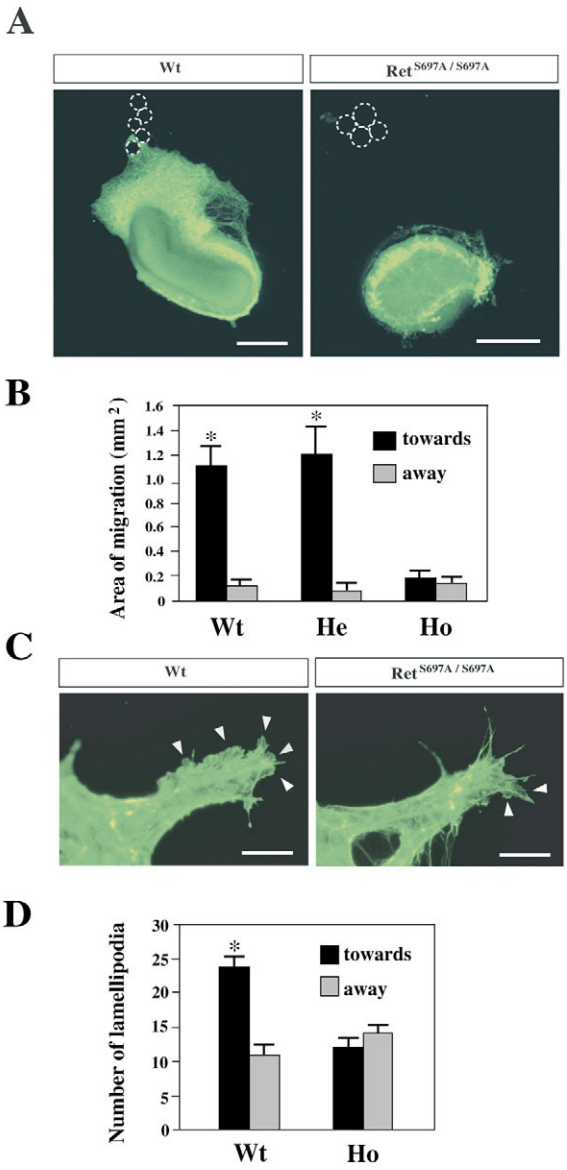


**Fig. 5. Impaired chemoattractant response of ENCCs in RET S697A mutant mice.** (**A**) Gut segments were dissected out from wild-type and homozygous RET S697A mutant mice at E12.5, and cultured on collagen gels with GDNF-soaked beads (dotted circles). After 4 days of culture, immunostaining with anti-PGP9.5 antibody was carried out to visualize ENCCs. Bars, 500 μm. (**B**) PGP9.5-positive area was measured in the quadrant facing GDNF-soaked beads (towards) and in the opposite quadrant (away). Six explants for each experiment. *$P$<0.01 compared with the distal quadrant. He, heterozygotes. (**C**) Lamellipodia formation in migrating ENCCs. F-actin was visualized with Alexa-phalloidin staining. Arrowheads indicate lamellipodia formation. Scale bars: 20 μm. (**D**) Quantitative analysis of lamellipodia formation. The number of lamellipodia was counted in the migrating cell front in the proximal and distal area. The number of lamellipodia per cell surface length (1 mm) was calculated. Six explants for each experiment. *$P$<0.01 compared with the distal quadrant.

It has been reported that GDNF functions as a chemoattractant for the ENCCs via RET activation, and that GDNF could control the migration of ENCCs in the developing gut (Iwashita et al., 2003; Natarajan et al., 2002; Young et al., 2001). To test whether the chemoattractant response to GDNF is affected by the S697A mutation, we cultured gut segments from wild-type and mutant mice together with GDNF-soaked beads on collagen gels. After 4 days, ENCCs were visualized by immunostaining with PGP9.5. Wild-type ENCCs migrated out massively from the gut segments towards GDNF-soaked beads, whereas migration of mutant ENCCs was extremely weak (Fig. 5A). To quantify the chemoattractant response to GDNF, PGP9.5-positive areas containing neurites and neural cells were measured in the proximal (towards) and distal (away) quadrants. In wild-type mice, the PGP9.5-positive area on the proximal quadrant towards the GDNF beads was significantly greater than in the distal quadrant. In the homozygous S697A mice, the proximal and distal areas were small and almost equal (Fig. 5B). These findings indicate that the S697A mutation impairs the GDNF-mediated migration of ENCCs.

As our previous results showed that the RET S697A mutation affects lamellipodia formation in cultured cell lines (Fukuda et al., 2002), we analyzed their formation in the migrating ENCCs towards GDNF-soaked beads. ENCCs were identified by immunostaining with anti-$p75^{NTR}$ antibody (data not shown). In ENCCs from wild-type mice, the number of lamellipodia in the proximal side was significantly greater than in the distal side. In the homozygous S697A mice, the number of lamellipodia showed no significant difference in the proximal and distal sides (Fig. 5C,D). These results suggest that GDNF-induced lamellipodia decrease in ENCCs from S697A mutant mice, resulting in their impaired migration.

## JNK signal is impaired in the RET S697A mutant mice and required for migration of ENCCs in cultured guts

Recently we reported that the RET S697A mutation specifically impairs the RAC1/JNK signaling pathway in cultured cells (Fukuda et al., 2002; Fukuda et al., 2005). To investigate whether JNK signal is also impaired in RET S697A mutant mice, DRG neurons, some of which are known to express RET, were collected from wild-type and mutant mice and stimulated with GDNF. As shown in Fig. 6A, ERK, AKT, p38MAPK and SRC were activated by GDNF in DRG neurons from both wild-type and homozygous S697A mice, whereas JNK activation by GDNF was specifically impaired in DRG neurons from mutant mice.

As it has been reported that the JNK signaling plays a role in cell migration or motility (Huang et al., 2004; Kawauchi et al., 2003; Meadows et al., 2004; Pedram et al., 2001), we hypothesized that the suppressed migration of ENCCs in the homozygous S697A mice is due to the impaired JNK signaling by the S697 mutant form of RET. To test whether JNK signaling was required for the chemoattractant response to GDNF, explant experiments were carried out in the presence of the JNK inhibitor SP600125. As expected, SP600125 markedly suppressed the chemoattractant response of ENCCs to GDNF (Fig. 6B,C).

To further analyze the importance of JNK signaling for migration of ENCCs in the developing gut, we cultured guts in vitro in the presence of various kinase inhibitors. Embryonic guts were dissected out at E11.5, and after 3 days of in vitro culture, ENCCs were visualized by PGP9.5 immunostaining. The PI3K inhibitor LY294002 severely impaired the migration of ENCCs in the cultured guts (Fig. 7C), whereas MEK1 inhibitor PD98059 showed little effect on their migration (Fig. 7B). Interestingly, in the guts cultured with the JNK inhibitor SP600125, migration of ENCCs was partly suppressed (Fig. 7D), which mimicked the impaired migration of ENCCs in the distal colon of the homozygous S697A embryos in vivo (Fig. 4F). Although the S697A mutation did not impair the SRC kinase activity (Fig. 6A), the SRC kinase inhibitor, SU6656, partly suppressed their migration (Fig. 7F). To quantify inhibitory effects, the ratios of the distance of ENCC migration to the colon length were measured and calculated. The ratios were 95±2% for control, and 93±2%, 35±3%, 75±3% and 75±2% in the presence of PD98059, LY294002, SP600125 and SU6656, respectively (Fig. 7).

Both the PKA inhibitor and the human RET S696A mutation showed similar biological effects in the cultured cell lines (Fukuda et al., 2002). To investigate the role of PKA in the migration of ENCCs, embryonic guts were cultured in the presence of the PKA inhibitor KT5720. KT5720 partly impaired the migration of ENCCs (the ratio of ENCC migration to the colon length was 78±6%), an effect similar to that of the JNK inhibitor (Fig. 7D,E). From these results, it seems likely that PKA controls the JNK signaling pathway downstream of RET via Ser697 phosphorylation, which is required for proper migration of ENCCs in the developing gut. Although we attempted the organ culture of the S697A mutant embryonic gut, mutant ENCCs did not migrate well even without kinase inhibitors under our experimental conditions (data not shown).

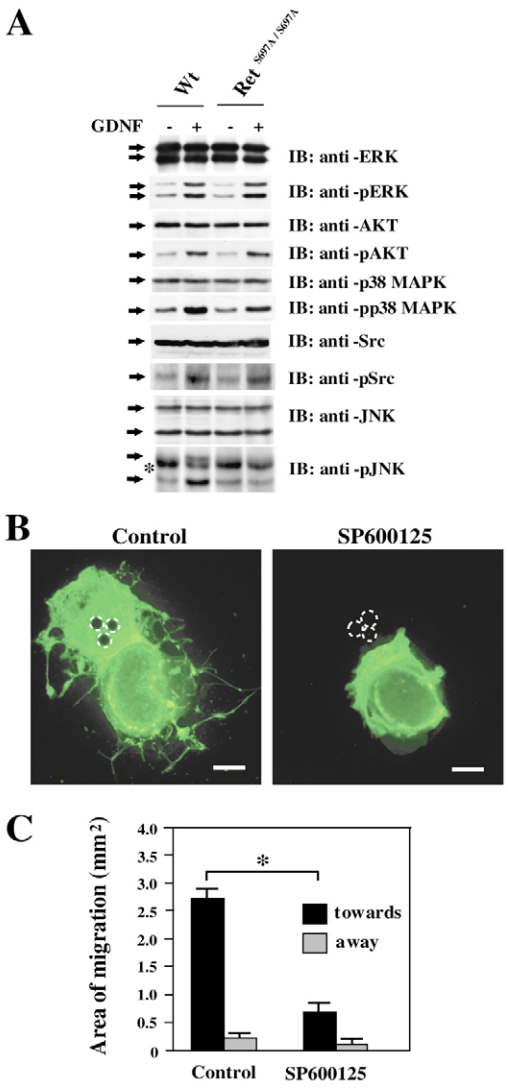


**Fig. 6. Impaired JNK signal in RET S697A mutant mice and impaired chemoattractant response of ENCCs by the JNK inhibitor.** (**A**) DRG was dissected wild-type and homozygous RET S697A mutant mice at postnatal day 5 and cultured for 72 hours. Lysates were prepared from DRG neurons untreated or treated with GDNF (20 minutes) and were analyzed by western blotting with the indicated antibodies. In the panel of anti-pJNK, an asterisk indicates a non-specific band. Two phosphorylated JNK bands were detected (arrows). (**B**) Gut segments were dissected out from wild-type mice at E11.5, and cultured with GDNF-soaked beads in the absence or presence of the JNK inhibitor SP600125. After 3 days of culture, immunostaining with anti-PGP9.5 was carried out. Scale bars: 500 μm. (**C**) PGP9.5-positive area was measured in the towards and away quadrants. Six explants for each experiment. *$P$<0.01 compared with control.

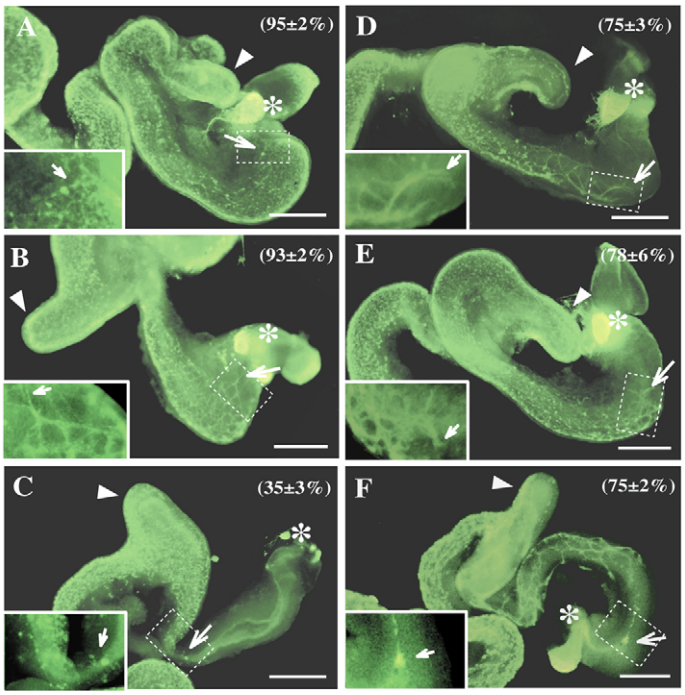


**Fig. 7. Inhibition of migration of ENCCs in gut culture by kinase inhibitors.** Embryonic guts were dissected out from wild-type mice at E11.5 and cultured in control medium (**A**), in medium with MEK1 inhibitor PD98059 (**B**), PI3K inhibitor LY294002 (**C**), JNK inhibitor SP600125 (**D**), PKA inhibitor KT5720 (**E**) and SRC kinase inhibitor SU6656 (**F**). After 3 days of culture, ENCCs were visualized by immunostaining with anti-PGP9.5 antibody. Each experiment was carried out at least twice using more than six embryonic guts. Arrows, the front of the migrating ENCCs; arrowheads, cecum; asterisks, terminus of the hindgut; bars, 200 μm. Insets show high-power views of the front of migrating ENCCs in dotted boxes of each panel. To quantify inhibitory effects, the ratios of the distance of ENCC migration to the colon length were measured and calculated. The ratios are shown in the upper right corner of each panel.

### S697A mutation does not impair proliferation, survival and neurite outgrowth of cultured ENCCs

In addition to the chemoattractant response, it is known that GDNF induces proliferation, survival and differentiation of cultured ENCCs. Thus, we investigated these biological responses using cultured ENCCs from wild-type and S697A mutant mice. As shown in Fig. 8A, both wild-type and mutant ENCCs showed remarkable neurite outgrowth in response to GDNF stimulation. In addition, after 3 days of culture, the neuron numbers did not show a significant difference between wild-type and mutant ENCC cultures (Fig. 8B). These findings suggest that proliferation, survival and differentiation of ENCCs were not significantly impaired by the S697A mutation.

### RET S697A mutation causes slight suppression of kidney development, but no abnormality in the sympathetic and parasympathetic neural system or in spermatogenesis

In addition to the ENS, the GDNF/RET signaling pathway also controls kidney development. The ratio of kidney weight to body weight in newborn wild-type mice was compared with mutant mice. The average kidney weight was 1.05%, 1.06% and 0.95% of total body weight in wild-type, heterozygous and homozygous mice, respectively. Thus, kidneys in the homozygotes were slightly but significantly reduced in size (Fig. 9A). Histological differences were not observed between wild-type and mutant kidneys (Fig. 9B).

*Ret*-deficient mice show an aberrant position of superior cervical sympathetic ganglion (SCG) and abnormal axonal projection (Enomoto et al., 2001). Thus, we analyzed the sympathetic system by whole-mount tyrosine hydroxylase immunostaining in newborn mutant mice. In the homozygous S697A mice, the size and location of SCG and chain ganglia appeared normal, and axons from ganglia projected properly (see Fig. S1 in the supplementary material). Moreover, no abnormalities were observed in the parasympathetic ganglia and motoneurons of the spinal cord or in the testis (see Fig. S1 in the supplementary material).

## DISCUSSION

Through a series of gene targeting experiments of *Ret*, *Gdnf* and *Gfrα-1* in mice, it has been revealed that RET signals play important roles in the development of the ENS and the kidney (Cacalano et al., 1998; Enomoto et al., 2001; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). The RET S697A mutant mice that were generated in the present study showed an obvious defect in ENS development, resulting in an absence of enteric ganglia in the distal colon, whereas the only kidney defect observed was a very slight reduction in size. Given that the RET S697A mutant mice lack a serine residue necessary for PKA-dependent modification of RET signaling, these findings suggest that this modification is more important in the development of the ENS than in that of the kidney.

In the nervous system, cAMP has been demonstrated to be a key regulator of neuronal survival, differentiation and regeneration via cAMP-mediated PKA activation (Cai et al., 1999). In addition, PKA appears to be constantly activated in the developing nervous system (Cai et al., 2001). Because GDNF has been shown to upregulate cAMP levels (Cai et al., 1999), it is reasonable to suggest that GDNF stimulation itself may trigger phosphorylation of RET S697. In the developing ENS, such a condition may be necessary for the PKA-dependent activity of RET, which regulates the activation of the JNK signaling pathway. Moreover, our result is consistent with a recent report which showed that the inhibition of JNK signaling does not affect ureteric bud branching in the developing kidney (Osafune et al., 2006).

Our data suggest that the PI3K pathway is a crucial signal in the migration of ENCCs. Inhibition of PI3K activity markedly suppressed the migration of ENCCs in the gut organ culture, whereas inhibition of MEK1 activity showed no significant effect (Fig. 7B,C). PI3K signaling induces activation of RHO family GTPases, including RHO, RAC and CDC42, which are essential for reorganization of the actin cytoskeleton, and thus for lamellipodia formation and cell motility (Govek et al., 2005; Watanabe et al.,

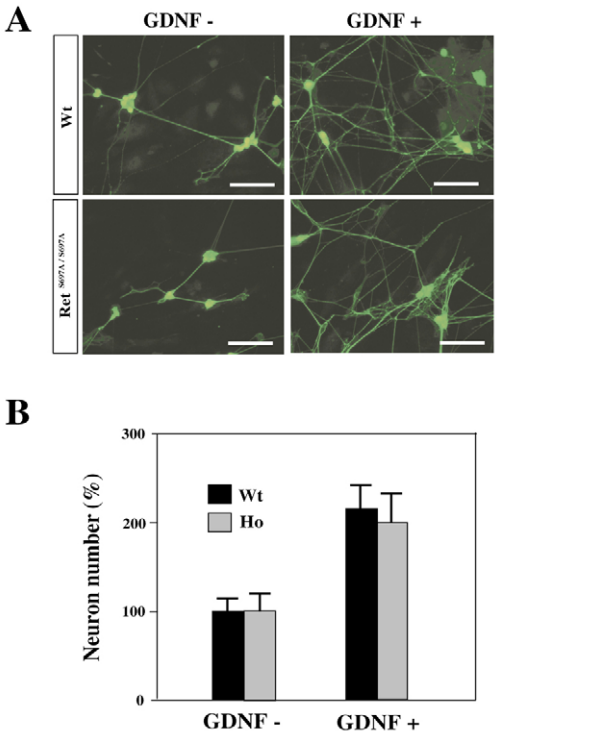


**Fig. 8. S697A mutation does not impair neurite outgrowth and proliferation/survival of cultured ENCCs.** (**A**) Neurite outgrowth of cultured ENCCs with GDNF stimulation. ENCCs were dissociated from E11.5 midgut and cultured for 3 days in the absence or presence of GDNF. Neurites were visualized by immunostaining with anti-PGP9.5 antibody. (**B**) Quantitative analysis of cultured ENCCs. After 3 days of culture in the absence or presence of GDNF, anti-PGP9.5-positive ENCCs were counted. The mean was calculated from ten views of each of three cultures.

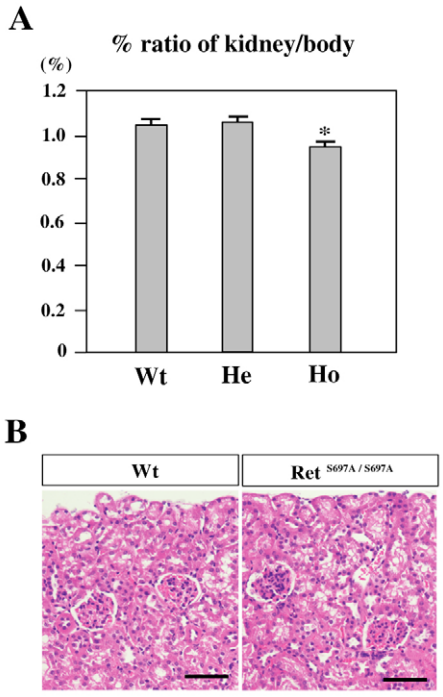


**Fig. 9. Reduced kidney size in RET S697A mutant mice.** (**A**) The weight of the kidney as a percentage of total body weight was determined in wild-type and mutant newborn mice (P3). Means and standard errors (error bars) were calculated from 23 wild-type, 55 heterozygous and 20 homozygous mice. *$P$<0.01 compared with wild-type mice. (**B**) Hematoxylin/Eosin staining of wild-type and mutant kidneys. Kidney of mutant mice at 2 months of age shows normal histology.

2005). In addition, we recently reported that AKT, a downstream effector of PI3K, plays an important role in actin reorganization and cell motility via phosphorylation of Girdin, a new AKT substrate (Enomoto et al., 2005). Thus, it is possible that migration of ENCCs is regulated via activation of these effector proteins downstream of PI3K.

Using a GDNF-mediated chemoattractant assay, in which ENCCs of the gut segments were cultured in collagen gels with GDNF-expressing cell pellets, it has been shown that treatment with the PI3K inhibitor severely suppressed the response of ENCCs to chemoattraction by GDNF, whereas treatment with the MEK1 inhibitor partly suppressed it (Natarajan et al., 2002). Although we did not observe any effect of the MEK1 inhibitor on ENCC migration in our gut culture system, this difference might be due to the different sensitivities of the different experimental systems, and/or to the different environmental conditions surrounding ENCCs in the whole-mount gut culture and in the chemoattractant assay. In accordance with the present result, we reported that mutant mice with the RET mutation Y1062F, which showed marked impairment of the PI3K/AKT signaling, developed aganglionosis in the whole ENS, supporting the view that the PI3K/AKT pathway is a critical signal for migration of ENCCs in the developing gut (Jain et al., 2004; Jijiwa et al., 2004; Wong et al., 2005). In addition, SRC kinase activity appears to play a role in the migration of ENCCs (Fig. 7F), although the S697A mutation did not impair its activity.

JNK is a serine/threonine protein kinase belonging to the family of MAPKs (Johnson and Lapadat, 2002). JNK is generally thought to play a role in inflammation, differentiation and apoptosis. However, recent studies demonstrated that JNK is important for the migration of several cell types, including keratinocytes, airway epithelial cells, vascular smooth muscle cells, leukocytes, cortical neurons and Schwann cells (Huang et al., 2004; Kawauchi et al., 2003; Meadows et al., 2004; Pedram et al., 2001). As well as the JNK activation with injury stress, JNK activation after stimulation with transforming growth factor β, interleukin 8, epidermal growth factor or platelet-derived growth factor can promote cell migration. JNK has been implicated in the control of the actin cytoskeleton and stress fibers in Drosophila embryos and mammalian cells (Kaltschmidt et al., 2002; Martin-Blanco et al., 2000; Zhang et al., 2003). In addition to the JNK-mediated phosphorylation of various well-studied apoptosis-related proteins and transcriptional factors, JNK directly phosphorylates a number of cytoskeleton-associated proteins and signaling molecules, including paxillin, spir, doublecortin, and microtubule-associated protein 1B and 2, which are suggested to play roles in cell migration (Chang et al., 2003; Gdalyahu et al., 2004; Huang et al., 2003; Kawauchi et al., 2003; Otto et al., 2000). In RET S697A mutant mice, JNK activation was specifically impaired, whereas both p38MAPK and ERK, which are also members of the MAPK family, were activated by GDNF. In addition, gut explant culture experiments showed that

chemoattractant response of ENCCs from S697A mice to GDNF and their lamellipodia formation were impaired and that the JNK inhibitor suppressed ENCCs migration. Although it is possible that suppressed migration of ENCCs in the homozygous S697A mice is due to undefined signals impaired by the S697A mutation, the present data strongly support the view that lack of JNK activation is responsible for the suppression of ENCC migration in the mutant mice.

We recently demonstrated that GDNF stimulation induced G2/M cell-cycle delay in MC(RET) cells but not in S696A cells (Fukuda et al., 2005). As this result suggested the possibility that RET S697A mutation affects the proliferation of ENS cells, we analyzed cell numbers in the ENS of the S697A mutant mice. As shown in Fig. 3, there was no significant difference in the total numbers of ENS cells in the whole small intestine and the proximal colon between wild-type and RET S697A mutant mice. Thus, the RET S697A mutation does not appear to affect cell proliferation in vivo. Moreover, we found that this mutation does not significantly affect proliferation, survival and neurite outgrowth of cultured ENCCs. ACHE-stained neural plexus and neural fibers showed normal number and size in the proximal mutant guts. A previous study reported a similar phenotype in the miRET$^{51}$ mice expressing only the long isoform of RET (de Graaff et al., 2001). The miRET$^{51}$ mice showed aganglionosis in the distal gut due to the delayed migration of ENCCs, although the ENS in the small intestine of mutant newborns was normal. As patients of Hirschsprung's disease also have aganglionosis in the distal colon, these mutant mice may provide useful models to study the molecular mechanisms of development of Hirschsprung's disease.

The RET tyrosine kinase is evolutionarily close to fibroblast growth factor receptor, vascular endothelial growth factor receptor, Kit and platelet-derived growth factor receptor. The 3′ halves of the juxtamembrane regions of RET and these other receptor tyrosine kinases share significant similarity, whereas the 5′ half of the juxtamembrane region, containing Y688 and S697, is unique in RET. Thus, the juxtamembrane region in RET may have a unique function. The juxtamembrane region in human RET contains one tyrosine residue, Y687 (corresponding to Y688 in mouse RET), which was reported as one of the autophosphorylation sites (Liu et al., 1996). We previously demonstrated that S696 is a putative phosphorylation site for PKA, and that lamellipodia formation impaired by the S696A mutation was rescued with an additional Y687F mutation, suggesting that S696 and Y687 phosphorylation leads to opposite biological effects (Fukuda et al., 2002). As Y687 and S696 are located closely, it seems likely that S696 phosphorylation suppresses Y687 phosphorylation via a conformational change of the juxtamembrane region. Interestingly, two Hirschsprung's disease-related *Ret* mutations (S690P and G691S) have been reported in this juxtamembrane region (Attie et al., 1995; Garcia-Barcelo et al., 2004). The computational prediction (NetPhosK: http://www.cbs.dtu.dk/services/NetPhosK/) suggests that both mutations may affect the probability of phosphorylation at Y687, supporting the idea that a signal from juxtamembrane region via Y687 phosphorylation plays a role in the ENS development.

This work was supported by grants-in-aid for Scientific Research (A), Scientific Research on Priority Area 'Cancer' and the 21st Century Center of Excellence Research from the Ministry of Education, Culture, Sports, Science, Technology of Japan to M.T., and NIH grant CA26767 to F.C and V.P.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/22/4507/DC1

**References**

**Airaksinen, M. S. and Saarma, M.** (2002). The GDNF family: signalling, biological functions and therapeutic value. *Nat. Rev. Neurosci.* **3**, 383-394.

**Attie, T., Pelet, A., Edery, P., Eng, C., Mulligan, L. M., Amiel, J., Boutrand, L., Beldjord, C., Nihoul-Fekete, C., Munnich, A. et al.** (1995). Diversity of RET proto-oncogene mutations in familial and sporadic Hirschsprung disease. *Hum. Mol. Genet.* **4**, 1381-1386.

**Barlow, A., de Graaff, E. and Pachnis, V.** (2003). Enteric nervous system progenitors are coordinately controlled by the G protein-coupled receptor EDNRB and the receptor tyrosine kinase RET. *Neuron* **40**, 905-916.

**Barone, M. V., Sepe, L., Melillo, R. M., Mineo, A., Santelli, G., Monaco, C., Castellone, M. D., Tramontano, D., Fusco, A. and Santoro, M.** (2001). RET/PTC1 oncogene signaling in PC Cl 3 thyroid cells requires the small GTP-binding protein Rho. *Oncogene* **20**, 6973-6982.

**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.

**Cai, D., Shen, Y., De Bellard, M., Tang, S. and Filbin, M. T.** (1999). Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. *Neuron* **22**, 89-101.

**Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregman, B. S. and Filbin, M. T.** (2001). Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate. *J. Neurosci.* **21**, 4731-4739.

**Chang, L., Jones, Y., Ellisman, M. H., Goldstein, L. S. and Karin, M.** (2003). JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins. *Dev. Cell* **4**, 521-533.

**Chiariello, M., Visconti, R., Carlomagno, F., Melillo, R. M., Bucci, C., de Franciscis, V., Fox, G. M., Jing, S., Coso, O. A., Gutkind, J. S. et al.** (1998). Signalling of the Ret receptor tyrosine kinase through the c-Jun NH2-terminal protein kinases (JNKS): evidence for a divergence of the ERKs and JNKs pathways induced by Ret. *Oncogene* **16**, 2435-2445.

**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.

**Edery, P., Lyonnet, S., Mulligan, L. M., Pelet, A., Dow, E., Abel, L., Holder, S., Nihoul-Fekete, C., Ponder, B. A. and Munnich, A.** (1994). Mutations of the RET proto-oncogene in Hirschsprung's disease. *Nature* **367**, 378-380.

**Encinas, M., Tansey, M. G., Tsui-Pierchala, B. A., Comella, J. X., Milbrandt, J. and Johnson, E. M., Jr** (2001). c-Src is required for glial cell line-derived neurotrophic factor (GDNF) family ligand-mediated neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway. *J. Neurosci.* **21**, 1464-1472.

**Enomoto, A., Murakami, H., Asai, N., Morone, N., Watanabe, T., Kawai, K., Murakumo, Y., Usukura, J., Kaibuchi, K. and Takahashi, M.** (2005). Akt/PKB regulates actin organization and cell motility via Girdin/APE. *Dev. Cell* **9**, 389-402.

**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., Heuckeroth, R. O., Golden, J. P., Johnson, E. M. and Milbrandt, J.** (2000). Development of cranial parasympathetic ganglia requires sequential actions of GDNF and neurturin. *Development* **127**, 4877-4889.

**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.

**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.

**Fukuda, T., Asai, N., Enomoto, A. and Takahashi, M.** (2005). Activation of c-Jun amino-terminal kinase by GDNF induces G2/M cell cycle delay linked with actin reorganization. *Genes Cells* **10**, 655-663.

**Garcia-Barcelo, M., Sham, M. H., Lee, W. S., Lui, V. C., Chen, B. L., Wong, K. K., Wong, J. S. and Tam, P. K.** (2004). Highly recurrent RET mutations and novel mutations in genes of the receptor tyrosine kinase and endothelin receptor B pathways in Chinese patients with sporadic Hirschsprung disease. *Clin. Chem.* **50**, 93-100.

**Gdalyahu, A., Ghosh, I., Levy, T., Sapir, T., Sapoznik, S., Fishler, Y., Azoulai, D. and Reiner, O.** (2004). DCX, a new mediator of the JNK pathway. *EMBO J.* **23**, 823-832.

**Govek, E. E., Newey, S. E. and Van Aelst, L.** (2005). The role of the Rho GTPases in neuronal development. *Genes Dev.* **19**, 1-49.

**Hellmich, H. L., Kos, L., Cho, E. S., Mahon, K. A. and Zimmer, A.** (1996). Embryonic expression of glial cell-line derived neurotrophic factor (GDNF) suggests multiple developmental roles in neural differentiation and epithelial-mesenchymal interactions. *Mech. Dev.* **54**, 95-105.

**Huang, C., Rajfur, Z., Borchers, C., Schaller, M. D. and Jacobson, K.** (2003). JNK phosphorylates paxillin and regulates cell migration. *Nature* **424**, 219-223.

**Huang, C., Jacobson, K. and Schaller, M. D.** (2004). MAP kinases and cell migration. *J. Cell Sci.* **117**, 4619-4628.
**Ichihara, M., Murakumo, Y. and Takahashi, M.** (2004). RET and neuroendocrine tumors. *Cancer Lett.* **204**, 197-211.
**Iwashita, T., Kruger, G. M., Pardal, R., Kiel, M. J. and Morrison, S. J.** (2003). Hirschsprung disease is linked to defects in neural crest stem cell function. *Science* **301**, 972-976.
**Jain, S., Naughton, C. K., Yang, M., Strickland, A., Vij, K., Encinas, M., Golden, J., Gupta, A., Heuckeroth, R., Johnson, E. M., Jr et al.** (2004). Mice expressing a dominant-negative Ret mutation phenocopy human Hirschsprung disease and delineate a direct role of Ret in spermatogenesis. *Development* **131**, 5503-5513.
**Jijiwa, M., Fukuda, T., Kawai, K., Nakamura, A., Kurokawa, K., Murakumo, Y., Ichihara, M. and Takahashi, M.** (2004). A targeting mutation of tyrosine 1062 in Ret causes a marked decrease of enteric neurons and renal hypoplasia. *Mol. Cell. Biol.* **24**, 8026-8036.
**Jing, S., Wen, D., Yu, Y., Holst, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R. et al.** (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* **85**, 1113-1124.
**Johnson, G. L. and Lapadat, R.** (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298**, 1911-1912.
**Kaltschmidt, J. A., Lawrence, N., Morel, V., Balayo, T., Fernandez, B. G., Pelissier, A., Jacinto, A. and Martinez Arias, A.** (2002). Planar polarity and actin dynamics in the epidermis of Drosophila. *Nat. Cell Biol.* **4**, 937-944.
**Kawauchi, T., Chihama, K., Nabeshima, Y. and Hoshino, M.** (2003). The in vivo roles of STEF/Tiam1, Rac1 and JNK in cortical neuronal migration. *EMBO J.* **22**, 4190-4201.
**Klein, R. D., Sherman, D., Ho, W. H., Stone, D., Bennett, G. L., Moffat, B., Vandlen, R., Simmons, L., Gu, Q., Hongo, J. A. et al.** (1997). A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. *Nature* **387**, 717-721.
**Kodama, Y., Asai, N., Kawai, K., Jijiwa, M., Murakumo, Y., Ichihara, M. and Takahashi, M.** (2005). The RET proto-oncogene: a molecular therapeutic target in thyroid cancer. *Cancer Sci.* **96**, 143-148.
**Lewandoski, M. and Martin, G. R.** (1997). Cre-mediated chromosome loss in mice. *Nat. Genet.* **17**, 223-225.
**Liu, X., Vega, Q. C., Decker, R. A., Pandey, A., Worby, C. A. and Dixon, J. E.** (1996). Oncogenic RET receptors display different autophosphorylation sites and substrate binding specificities. *J. Biol. Chem.* **271**, 5309-5312.
**Maeda, K., Murakami, H., Yoshida, R., Ichihara, M., Abe, A., Hirai, M., Murohara, T. and Takahashi, M.** (2004). Biochemical and biological responses induced by coupling of Gab1 to phosphatidylinositol 3-kinase in RET-expressing cells. *Biochem. Biophys. Res. Commun.* **323**, 345-354.
**Martin-Blanco, E., Pastor-Pareja, J. C. and Garcia-Bellido, A.** (2000). JNK and decapentaplegic signaling control adhesiveness and cytoskeleton dynamics during thorax closure in Drosophila. *Proc. Natl. Acad. Sci. USA* **97**, 7888-7893.
**Meadows, K. N., Bryant, P., Vincent, P. A. and Pumiglia, K. M.** (2004). Activated Ras induces a proangiogenic phenotype in primary endothelial cells. *Oncogene* **23**, 192-200.
**Meng, X., Lindahl, M., Hyvonen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M. et al.** (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287**, 1489-1493.
**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.
**Murakami, H., Iwashita, T., Asai, N., Iwata, Y., Narumiya, S. and Takahashi, M.** (1999). Rho-dependent and –independent tyrosine phosphorylation of focal adhesion kinase, paxillin and p130Cas mediated by Ret kinase. *Oncogene* **18**, 1975-1982.
**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.
**Osafune, K., Takasato, M., Kispert, A., Asashima, M. and Nishinakamura, R.** (2006). Identification of multipotent progenitors in the embryonic mouse kidney by a novel colony-forming assay. *Development* **133**, 151-161.
**Otto, I. M., Raabe, T., Rennefahrt, U. E., Bork, P., Rapp, U. R. and Kerkhoff, E.** (2000). The p150-Spir protein provides a link between c-Jun N-terminal kinase function and actin reorganization. *Curr. Biol.* **10**, 345-348.
**Pachnis, V., Mankoo, B. and Costantini, F.** (1993). Expression of the c-ret proto-oncogene during mouse embryogenesis. *Development* **119**, 1005-1017.
**Pedram, A., Razandi, M. and Levin, E. R.** (2001). Natriuretic peptides suppress vascular endothelial cell growth factor signaling to angiogenesis. *Endocrinology* **142**, 1578-1586.
**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.
**Romeo, G., Ronchetto, P., Luo, Y., Barone, V., Seri, M., Ceccherini, I., Pasini, B., Bocciardi, R., Lerone, M., Kaariainen, H. et al.** (1994). Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. *Nature* **367**, 377-378.
**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.
**Treanor, J. J., Goodman, L., de Sauvage, F., Stone, D. M., Poulsen, K. T., Beck, C. D., Gray, C., Armanini, M. P., Pollock, R. A., Hefti, F. et al.** (1996). Characterization of a multicomponent receptor for GDNF. *Nature* **382**, 80-83.
**van Weering, D. H. and Bos, J. L.** (1997). Glial cell line-derived neurotrophic factor induces Ret-mediated lamellipodia formation. *J. Biol. Chem.* **272**, 249-254.
**Watanabe, T., Noritake, J. and Kaibuchi, K.** (2005). Regulation of microtubules in cell migration. *Trends Cell Biol.* **15**, 76-83.
**Wong, A., Bogni, S., Kotka, P., de Graaff, E., D'Agati, V., Costantini, F. and Pachnis, V.** (2005). Phosphotyrosine 1062 is critical for the in vivo activity of the Ret9 receptor tyrosine kinase isoform. *Mol. Cell. Biol.* **25**, 9661-9673.
**Young, H. M. and Newgreen, D.** (2001). Enteric neural crest-derived cells: origin, identification, migration, and differentiation. *Anat. Rec.* **262**, 1-15.
**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.
**Zhang, L., Wang, W., Hayashi, Y., Jester, J. V., Birk, D. E., Gao, M., Liu, C. Y., Kao, W. W., Karin, M. and Xia, Y.** (2003). A role for MEK kinase 1 in TGF-beta/activin-induced epithelium movement and embryonic eyelid closure. *EMBO J.* **22**, 4443-4454.