Supplemental Data Materials and Methods

HSCR family

A Turkish HSCR family consisting of six members spanning three generations was included in the present study. There was no consanguinity. Histological examination of surgical resection materials was used for the diagnosis of HSCR and to determine the extent of aganglionosis. All the affected members show no other syndrome. The father (I1) and the mother (I2) of the index case (II2) did not show any HSCR-like symptoms nor have experienced persistent constipation. However, two I2’ sisters died in the neonatal period although the medical records on their deaths are not available to ascertain if they were also HSCR patients and died of bowel obstruction.

DNA isolation

Venous blood (3 ml) were collected into an EDTA tube and stored in -80°C freezer until DNA was isolated. DNA was extracted from blood using QIAamp DNA Blood kit following manufacturer’s protocol (QIAGEN, Clifton Hill, Victoria Australia). All affected and unaffected members of the family gave informed consent for molecular analysis.

Mutation detection

Using PCR amplification and direct sequencing, we screened all exons of the RET, GDNF, EDNRB, and EDN3 genes including intron/exon boundaries for mutations and polymorphisms in
all the family members. PCR products were screened for mutations by direct sequencing using a dye terminator cycle sequencing kit (ABI PRISM® Big Dye™ Terminator v 2.0 Cycle sequencing kit, Applied Biosystems, Foster City, CA) and an ABI 3100 automated sequencer (Applied Biosystems).

**Mutant RET construct**

The cDNA clone encoding the wild-type human RET9 in the pcDNA3.1 (+) vector (Invitrogen, CA USA) was a gift from Dr. L. Mulligan (Department of Paediatrics, Queen’s University, Kingston, Canada). The 5 bp deletion was generated by site-directed mutagenesis (QuickChange XL Site-Directed Mutagenesis Kit, Stratagene) using the following primers 5’-ATC GCA GCC GCT GTC CTC CTT CAT CGT CTC-3’ and 5’-GAG ACG ATG AAG GAG GAC AGC GGC TGC GAT-3’. The mutated construct was verified by sequencing throughout the insert.

**Cell transfection**

HTB11 neuroblastoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL) at 37°C in atmospheric air with 5% CO₂. Cells (8x10⁴) were seeded onto a 6-wells plate 24 hours before transfection with 0.5 μg of either normal RET or mutant RET constructs using Lipofectamine 2000 (Invitrogen, CA USA). Transfected cells were harvested after 24 hours for cell lysate or total RNA isolation.
**RT-PCR analysis**

Total RNA was isolated using Trizol (Life Technologies, Rockville, MD). First strand cDNA synthesis was performed on 2 μg of RNA using oligo(dT) as reverse primer (SuperScript Preamplification System, Life Technologies). PCR amplification was performed in 25 μl of standard buffer containing primers (25 μmol/L each), 3 μl cDNA, 1 unit AmpliTaq DNA polymerase (ABI), MgCl₂ (25 mmol/L), 5% DMSO and dNTP (10 mmol/L). Primers used were: 5’-ACA CCA AGG CCC TGC GGC G-3' and 5’-GGA AGG TCA TCT CAG CTG AG-3’. After the initial denaturation at 95°C for 8 minutes, PCR was performed for 35 cycles as follow:- 95°C, 1 minute; 54°C, 45 seconds; 72°C, 45 seconds. A final 10 minutes extension at 72°C was added. RT-PCR products were digested with restriction endonuclease EarI, and digested products were analyzed by electrophoresis in a 2% agarose gel.

**Western blotting**

Cell pellet from each well of a 6-wells plate was lysed by trituration in 100 μl of diluted lysis buffer (10%; Cell Signaling Technology, Inc., MA USA). 25 μg of protein lysate was mixed with loading dye (6 x loading dye: 0.35 mol/L Tris-HCl, pH 6.8; 10.28% (w/v) SDS; 36% (v/v) Glycerol; 5% β-mercaptoethanol; 0.012% (w/v) bromophenol blue), denatured at 95°C for 10 minutes before being analyzed on a 8% denaturing polyacrylamide gel. The samples were
electro-transferred to Microporous polyvinylidene difluoride (PVDF) membrane (Immobilon-P Transfer Membrane, Millipore, Bedford, MA USA) at 4°C overnight. Membrane was blocked with 5% non-fat milk in TBS-T for 1.5 hour at room temperature, and then incubated with a rabbit polyclonal antibody that recognized the N-terminal region of human RET protein (anti-RET-N; Ret H-300, 1:800 dilution in blocking solution; Santa Cruz Biotechnology, CA USA) for 1 hour at RT. After TBS-T washings (3x10 minutes each; room temperature), the membrane was incubated with HRP-conjugated anti-rabbit antibody (Zymed Laboratories, Inc., CA USA) for 1 hour at room temperature. Membrane was washed and signal was developed using ECL Plus Western blotting Detection System and Hyper ECL film (Amersham Biosciences).

**Immuno-fluorescence**

HTB11 neuroblastoma cells were seeded onto 24 mm² cover slip in 6-wells plate 24 hours before transfection with 1.5 or 0.25 μg of either wild-type RET or mutant RET constructs using Lipofectamine 2000 (Invitrogen, CA USA). 24 hours after transfection, cells on the cover slips were washed once with PBS before being fixed in formaldehyde/methanol as follow:- 10 minutes in 3.6% formaldehyde in PBS; rinse in methanol before incubation with methanol for 20 minutes; washed with PBS. Fixed cells were blocked with blocking solution (1% BSA in PBS) at room temperature for 1.5 hour before incubation with primary antibody for 16 hours at 4°C.
Cover slips were washed with blocking solution (3 times, 5 minutes each) and incubated with appropriate TRITC-conjugated secondary antibodies for 1 hour at room temperature. After washing with blocking solution (3 times, 5 minutes each) and PBS (1 times, 5 minutes), cover slips were dehydrated by passing through a graded series of ethanol before completely dried in air. Dried cover slips were mounted in DAPI-containing anti-fade mounting medium (Vector Laboratories, Inc. CA, USA). Immuno-fluorescent stained cells were examined with an epifluorescence microscope (Nikon). Images were captured with digital camera (Nikon) and photos were compiled using Adobe Photoshop 7. Rabbit polyclonal antibodies that recognized the N- and C-terminal (anti-RET-C) regions of human RET protein were used to detect the normal RET and the truncated RET proteins in the transfected HTB11 cells (anti-RET-N; Ret H-300, 1:100 dilution in blocking solution; Santa Cruz Biotechnology, CA USA; anti-RET-C; Ret C-19, 1:100 dilution in blocking solution; Santa Cruz Biotechnology, CA USA). TRITC-conjugated goat-anti-rabbit antibody (1:100 dilution in blocking solution; Zymed Laboratories, Inc., CA USA) was used as secondary antibody.

**Purification of biotinylated cell surface proteins and Western blotting**

Biotinylation and purification of cell surface protein was performed on HTB11 cells transfected either with mutant \textit{RET} or empty vector using Cell Surface Biotinylation and Purification Kit
(PIERCE, IL USA) following manufacturer’s protocol. In brief, transfected cells were biotinylated and lysed. Cell lysate was added to the avidin-sepharose purification column, and the first flow-through was collected as cytoplasmic protein fraction. The bound biotinylated cell membrane proteins were eluted from the column with SDS-PAGE buffer containing 50 mM DTT according to manufacturer’s protocol. 18 μg of cell membrane protein or cytoplasmic protein was analyzed on a 10% denaturing polyacrylamide gel, and electro-transferred to Microporous polyvinylidene difluoride (PVDF) membrane. Western blotting analysis was performed using a rabbit polyclonal antibody that recognized the N-terminal region of human RET protein (anti-RET-N) as described above. Signal was developed using ECL Plus Western blotting Detection System and Hyper ECL film (Amersham Biosciences).