Oncogene (1998) 17, 3065–3070

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SHORT REPORT

Genomic structure of the gene for the SH2 and pleckstrin homology domain-containing protein *GRB10* and evaluation of its role in Hirschsprung disease

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Hirschsprung disease (HSCR), or congenital aganglionic megacolon, is the most frequent cause of congenital bowel obstruction. Germline mutations in the *RET* receptor tyrosine kinase have been shown to cause HSCR. Mice that carry null alleles for *RET* or for its ligand, glial cell line-derived neurotrophic factor (*GDNF*), both exhibit complete intestinal aganglionosis and renal defects. Recently, the Src homology 2 (SH2) domain-containing protein Grb10 has been shown to interact with RET *in vitro* and *in vivo*, early in development. We have confirmed the map location of *GRB10* on human chromosome 7, isolated human BACs containing the gene, elucidated its genomic structure, isolated a highly polymorphic microsatellite marker adjacent to exon 14 and scanned the gene for mutations in a large panel of HSCR patients. No evidence of linkage was detected in HSCR kindreds and no mutations were found in patients. These data suggest that while *GRB10* may be important for signal transduction in developing embryos, it does not play an obvious role in HSCR.

Keywords: Hirschsprung disease; GRB10; GRB-IR; *RET* receptor tyrosine kinase; mutation detection; genomic structure; mapping

Congenital aganglionic megacolon, commonly known as Hirschsprung disease (HSCR), is associated with a lack of intrinsic ganglion cells in the myenteric and submucosal plexuses along variable lengths of the gastrointestinal tract (Holschneider, 1982). Enteric ganglion cells are derived primarily from the vagal neural crest. Thus, HSCR, like other disorders whose affected tissues are of neural crest origin, is best characterized as a neurocristopathy, albeit one that frequently co-occurs with additional phenotypes affecting neural crest-derived tissues (Bolande, 1997; Martucciello, 1997). HSCR is relatively common, with an incidence of approximately 1 in 5000 live births (Spouge and Baird, 1985).

Evidence that HSCR susceptibility has a large genetic component has come from pedigrees segregating HSCR as an incompletely penetrant autosomal dominant trait and formal segregation analysis supporting the hypothesis of dominant inheritance in at least 20% of cases, with the remainder of cases explainable by recessive or multigenic inheritance (Badner *et al*., 1990; Bodian and Carter, 1963). Subsequently, genetic mapping of HSCR to the pericentromeric region of chromosome 10 in a subset of families (Angrist *et al*., 1993; Lyonnet *et al*., 1993, and to chromosome 13q22 in a large Mennonite kindred (Puffenberger *et al*., 1994a) was reported.

In 1994, mutations in HSCR patients were described in several genes, most notably in the *RET* receptor tyrosine kinase (Edrey *et al*., 1994; Romeo *et al*., 1994) and in the G-protein coupled endothelin-B receptor (*EDNRB*; Puffenberger *et al*., 1994a). Mutations have since been reported in *EDNRB*'s physiological ligand, endothelin 3 (Hofstra *et al*., 1996; Kusafuka *et al*., 1996, 1997. Mouse knockout phenotypes for these genes, and for RET's ligand GDNF, support roles for them in the development of the enteric nervous system (Jing *et al*., 1996; Moore *et al*., 1996; Sanchez *et al*., 1996; Schuchardt *et al*., 1994), as mice carrying null alleles in each case exhibit intestinal aganglionosis. However, given the high likelihood that mutations in *RET* and the endothelins account for no more than 50% of all cases of HSCR (Attie *et al*., 1995; Chakravarti, 1996; Seri *et al*., 1997), substantial additional genetic factors remain to be discovered.

The importance of RET in the development of neural crest derivatives, and HSCR in particular, suggested that downstream components of the RET signal transduction pathway might also predispose to HSCR susceptibility. Recently, the SH2 domain-containing protein Grb10 (Ooi *et al*., 1995) was found to be a constituent of this pathway. Using mouse Ret as bait in a yeast two-hybrid assay, Pandey *et al*. (1995) isolated Grb10 as prey from an embryonic day 10.5 expression library. *In vitro* binding studies and co-immunoprecipitation experiments confirmed these results. Among other prey, Grb10 was also obtained in a Ret two-hybrid screen by another group (Durick *et al*., 1996). Using the cytoplasmic domain of human RET as bait, we conducted another two-hybrid screen of a mouse embryonic day 11 cDNA library. Among the 21 positives selected for signal strength in the β-galactosidase assay was a mouse cDNA corresponding to the SH2 and pleckstrin homology domains of Grb10 (Angrist, 1996). Thus, in several independent two-hybrid screens of early embryonic libraries, the SH2 domain of Grb10 was found to interact with the intracellular domain of Ret.

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Received 16 April 1998; revised 18 June 1998; accepted 18 June 1998

GRB10 was also viewed by us as a candidate for HSCR susceptibility for reasons other than its association with *RET*. Its expression in the early embryo is consistent with a role in enteric nervous system development (Angrist, 1996; Okamoto and Ueta, 1967; Pandey *et al*., 1995; Webster, 1973). In addition to an SH2 domain at its carboxy terminus, Grb10 also contains a pleckstrin homology (PH) domain of ~100 amino acids (Margolis, 1994; Ooi *et al*., 1995). PH domains have been implicated in cellular signaling and cytoskeletal organization, especially in those molecules associated with cell membranes (Shaw, 1996).

The sequence that codes for the PH domain that is present in the so-called hGrb-IRβ/Grb10 and hGRB10/IR-SV1 isoforms of GRB10 (Frantz *et al*., 1997; O'Neill *et al*., 1996) is part of a larger region in the coding sequence that bears striking identity to the *C. elegans* cosmid F10E9.6 and the gene it contains, *mig-10*. *Mig-10* is critical for migration of several groups of neurons. What these neurons have in common is that, like presumptive enteric neurons in the mammal, they all undergo long-range migrations along the antero-posterior axis. Additionally, mutant *mig-10* animals all have shortened posterior excretory canals. Manser and Wood (Manser *et al*., 1997; Manser and Wood, 1990) have suggested that the pleiotropic nature of this mutation may arise as the result of a defect in a component of the basal lamina that is critical for both canal outgrowth and neuronal migration. Thus, given the failure of excretory cell migration and the presence of non-cell autonomous defects, pleiotropy, incomplete penetrance as well as the potential involvement of the extracellular matrix, it is clear that *mig-10* mutant and HSCR phenotypes share several salient genetic features.

In order to assess *GRB10*'s role in HSCR and further characterize the gene, we have confirmed its map location, isolated bacterial artificial chromosomes (BACs) containing human genomic *GRB10* sequence, determined its intron-exon boundaries, and identified a highly polymorphic microsatellite in intron 13. We have also screened a panel of HSCR patients and families for linkage to chromosome 7p by genotyping microsatellite polymorphisms and for mutations in *GRB10* by nucleotide sequencing. No evidence of linkage was observed and no mutations were detected in HSCR patients.

Initially, *GRB10* was mapped to human chromosome 7 by somatic cell hybrid mapping. Using unique primers from the *GRB10*-derived expressed sequence tag (EST) c-1kf03 (GenBank accession no. Z43779), a 103 bp product was PCR-amplified essentially as described (Angrist *et al*., 1995a; 53° annealing temperature). Primers used were: C1KF03.F: 5′-GAGAGGTGCTTGGAAGACCAT-3′ and CIKF03.R: 5′-AGAACTCGTATTTTGCGTAAT-3′. Chromosomal mapping was accomplished by PCR genotyping against the National Institute of General Medical Sciences (NIGMS) human × rodent somatic cell hybrid mapping panel 2 (Coriell Institute for Medical Research, Camden, NJ, USA. Regional chromosomal localization was performed using the Stanford G3 Radiation Hybrid Mapping Panel as DNA template, as previously described (Angrist *et al*., 1995b). Both somatic cell hybrid and radiation hybrid mapping experiments yielded a single, unambiguous band. Data were submitted to the Stanford RH Web Server (http://www-shgc.stanford.edu/rhserver2/rhserver_form.html) for map analysis. RH mapping refined the gene's position to within 35.2 centiRays (≈700 kb) of D7S2467 and 52.2 centiRays (≈1050 kb) of D7S2422 by radiation hybrid mapping. The best location is depicted in Figure 1. Based on a recent integrated physical and genetic map of human chromosome 7 (Bouffard *et al*., 1997), the cytogenetic location of *GRB10* can be deduced to be 7p11.2–7p12. This map placement is in close agreement with other recently reported fluorescence *in situ* hybridization and radiation hybrid mapping data from two independent groups (Dong *et al*., 1997; Jerome *et al*., 1997).

Genomic DNA containing *GRB10* was obtained by screening a BAC library with a fragment derived from the cDNA clone obtained in an earlier two-hybrid screen (Angrist, 1996) and that most closely resembled the sequence of human *GRB-IR* (Liu and Roth, 1995). A 1164 bp *Spe*I fragment (nucleotides 661–1824) derived from this clone was excised from a two-hybrid system prey vector (pGAD10, a gift from Dr Stanley Fields, University of Washington, Seattle, WA), cleaned using the Wizard DNA Clean-up System (Promega, Madison, WI, USA) and used to probe the Research Genetics (Huntsville, AL) Bacterial Artificial Chromosome (BAC) Library filters. Hybridizations, isolation of BAC DNA and manual sequencing were performed as described (Angrist *et al*. 1998). The gene was found to contain at least 16 exons (Figure 1, Table 1). Exon and approximate intron sizes are listed in Table 1. The genomic boundaries of human *GRB10* are estimated to encompass at least 47 kb.

A potentially polymorphic dinucleotide repeat ($[TG]_{25}TAA[GA]_2G[TG]_6$) was detected 123 bp upstream of the 5′ end of exon 14. We amplified this sequence using radioactively end-labeled primer in 50 CEPH control individuals and 161 HSCR patients and their families. Primers used were: GRBIRCAR.F1: 5′-GTCTTGGT-GCTTGCCTGGTGTG-3′ and GRBIRCAR.R1: 5′-GGCTGTCACGGAGGAGAAAAAG-3′. Amplification conditions were as described (Puffenberger *et al*., 1994b), except the annealing temperature was 56°C and the Mg^{2+} concentration was 0.75 mM. Allele sizes and frequencies of the microsatellite marker, designated GRB10-CA_n, are listed in Table 2, with a heterozygosity of 0.82 determined empirically in CEPH control individuals. When this marker was tested for linkage in HSCR families and sib pairs, no evidence of linkage or increased allele sharing among affected individuals was detected. In order to assess allele sharing among affecteds, we utilized the nonparametric linkage (NPL) test, as implemented in the program GENEHUNTER (Kruglyak *et al*., 1996). This analysis yielded NPL Z scores of -0.42 ($P=0.69$) for nine large kindreds, -0.38 ($P=0.64$) for 30 sib pairs, and -0.55 ($P=0.71$) for all families segregating HSCR combined. Additionally, we obtained maximum two-point parametric lod scores for HSCR versus GRB10-CA_n of -7.3 for nine large families, -5.3 for 30 sib pairs, and -12.6 for the combined family data. Parametric analyses assumed a rare autosomal dominant gene ($P=0.001$) and sex-dependent reduced penetrance in males (58%) and females (29%), as described in Angrist *et al*. (1993).

After obtaining informed consent, a panel of 85+ biopsy-proven HSCR patients was screened for mutations in *GRB10*. Patients were chosen without regard to segment length or accompanying phenotypes; the panel used here closely resembled that used in our previous non-Mennonite HSCR studies (Angrist 1995a, 1996) and represented the broad spectrum of segment length and associated manifestations of neural crest disorders. Using the intron-exon boundary information obtained from direct BAC

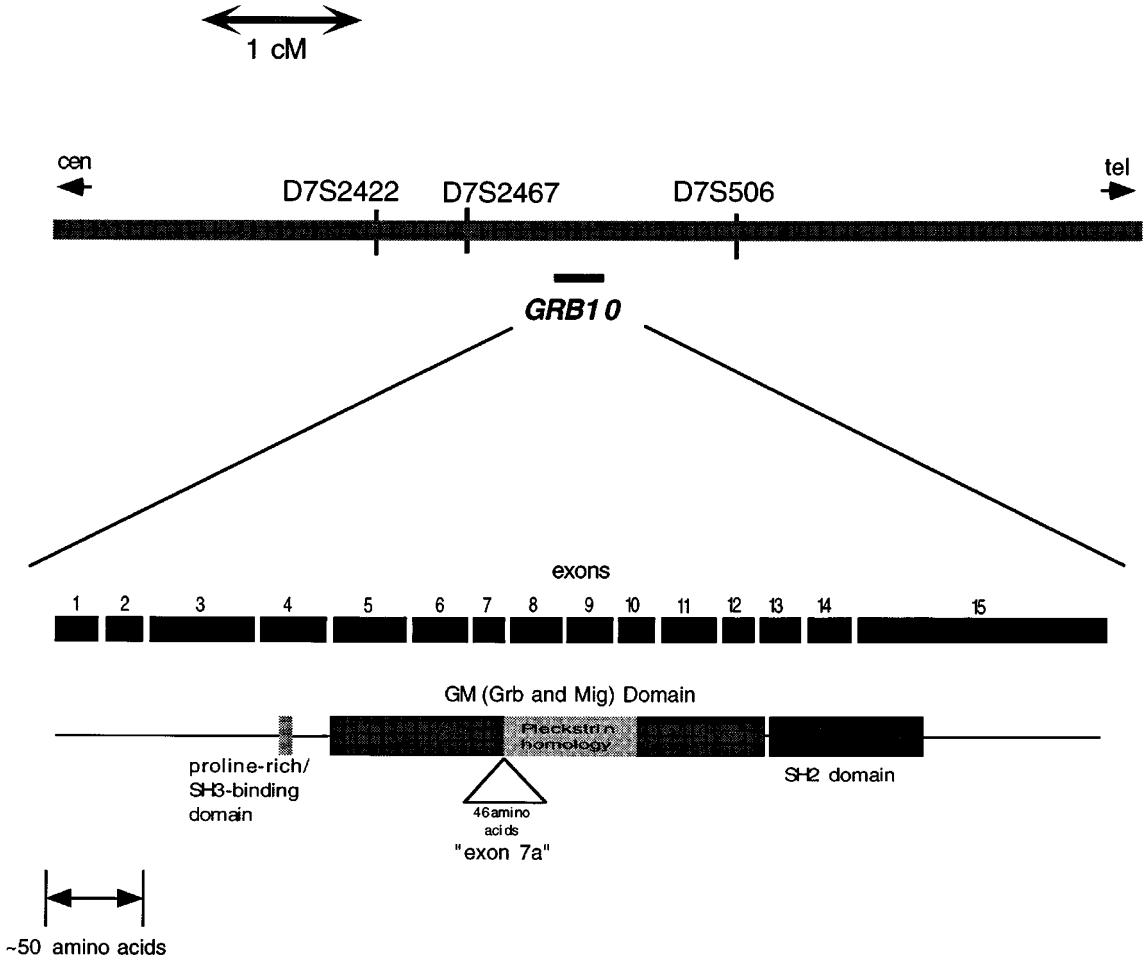

Figure 1 (Top) Genetic map of human chromosome 7p11.2-p12. Horizontal bar indicates most likely location of *GRB10* based on radiation hybrid mapping; marker locations are from Dib *et al.* (1996). (Bottom) Schematic representation of GRB10 exons. The relative size of each exon is indicated by the size of its box; intronic sequence is not represented. Below the exons, the corresponding protein domains are depicted. 'Exon 7a' is presumed to exist 3′ of exon 7 based on reports of another *GRB10*/*GRB-IR*/*hGRB10γ* isoform (Frantz *et al.*, 1997; O'Neill *et al.*, 1996). The top and bottom portions of the figure are not drawn to the same scale

Table 1 Human *GRB10* genomic structure

Exon	*Size (bp)*	*Position in cDNA[a] (nt)*	*Intron size (kb)*	*Splice acceptor*	*Splice donor*
1	82	1–82	~2.9	5′ UTR	TACTACCAG**gt**atgggcga
2	88	83–170	~6.0	ttttttt**ag**GACAAGGTG	ATCACCAGC**gt**aaggaatg
3	223	171–393	~5.7	ttctgtgc**ag**AGGATGATG	CGCTGTCAG**gt**aggtccag
4	142	394–535	~1.6	tgccttcc**ag**GCGCCTTCA	GCAAAGCAG**gt**gagtgtgc
5	157	536–692	~2.2	tgtgttgc**ag**GATGTTAAA	TAGGATTAG**gt**agggaacc
6	118	693–810	~1.2	gcccctgc**ag**AGAGAGGTG	AATCCCATG**gt**gagtctta
7	69	811–879	~3.5	attttctt**ag**AATTTCTTC	CTTTTGCAG**gt**actgggcc
7A[b]	138	880–1017	<3.3	tcttcaac**ag**AATTTTCTG	ACTTCAAAG**gt**gagcttta
8	111	880–990	~2.0	gttgtcac**ag**GAACCCAGA	TGCATAAAG**gt**acccacga
9	99	991–1089	~6.7	cctcctgt**ag**CCAAACAAA	CTCCTCAAG**gt**atgtgaca
10	78	1090–1167	~1.1	tcctctcc**ag**TATGGAATG	ACGCCAGTG**gt**aagtaaag
11	117	1168–1284	~1.1	tccttttc**ag**GCCAGTGTC	CCCTGGAGG**gt**aaggcccc
12	67	1285–1351	~0.3	tcttttgc**ag**AAGCGAAGC	TAAGTACAG**gt**aaacaggg
13	88	1352–1439	~9.0	ccccccac**ag**TGATTCACA	CGTGGATGG**gt**aaggaacc
14	94	1440–1533	~2.0	ccttcccc**ag**GCTTTTTCT	ATCTTACC**Tgt**aagtattg
15	539	1534–2072	–	gtctctcc**ag**TGCGAGGAC	3′ UTR

[a]cDNA sequence from Liu and Roth (1995). [b]Position based on additional *GRB10* splice variants reported by others (Frantz *et al.*, 1997).

sequencing, primers were designed to PCR amplify the 16 exons of human *GRB10* (Table 3). PCR amplification and sequencing were carried out as described (Angrist *et al*., 1998). Beyond the microsatellite marker, several sequence variants were found in *GRB10* in patients (Table 4). All were silent changes, none of which are expected to disrupt splicing or otherwise interfere with proper *GRB10* function, although this cannot be demonstrated conclusively in the absence of functional studies. Interestingly, two of the three intronic variants altered two closely positioned but non-consecutive nucleotides (Table 4), suggesting that *GRB10* might be undergoing gene conversion. We consider this to be a reasonable hypothesis, given that these sequence changes reside outside canonical motifs necessary for proper splicing (Senapathy *et al*., 1990) and that there are at least three other extant genes in the genome closely related to *GRB10* (Daly *et al*., 1996; Margolis, 1994).

As reported here, *GRB10* was found to span a genomic region of ~47 kb and to include at least 16 exons (Figure 3). These exons encompass all published *GRB10* splice variants and include both the unique N-terminus of *hGRB-IR* and the full-length PH domain of *hGRB-IRβ* (also called *GRB-IR^{PH}* and *hGRB10/IR-SV1*; Frantz *et al*., 1997; Liu and Roth, 1995, 1998; O'Neill *et al*., 1996) Each intron-exon boundary listed

Table 2 Allele sizes and frequencies for microsatellite marker GRB10-CA_n in 50 unrelated individuals

Allele	*Size (bp)*	*Frequency*
1	224	0.01
2	226	0.05
3	228	0.01
4	230	0.34
5	232	0.09
6	234	0.02
7	236	0.07
8	238	0.05
9	240	0.13
10	242	0.14
11	244	0.09

Table 3 Primers used for genomic amplification of human *GRB10* exons

Exon	*Exon size (bp)*	*Product size (bp)*	*Annealing temperature (°F)*	*Forward primer (5′→3′)*	*Distance to 5′ end of exon (bp)*	*Reverse primer (5′→3′)*	*Distance from 3′ end of exon (bp)*
1	82	213	58	GGCTTGGCTTCTCACAGTCTG	58 to ATG	ATCTATGGCTGGTGGCGACAT	62
2	88	159	55	TTCTGCTGCGGTCCTGTTTTT	5	CAAGCTAGAACTGGGAGTGT	24
3	223	321	56 + DMSO	CCTGATCACCAAGATGTACA	63	GAGGCCTGGACCTACCTG	0
4	142	298	58	GTGTAAGGCTGGGTCAT	36	ACTTCCCGCCCTTCTTCC	84
5	157	242	55	TTTCTTGAAAGCCCGAAGTT	40	AGGTGCCAATCCTGTTCTGA	12
6	118	248	53	TACCATGAATTTCCCACCTGT	32	TGATACTATGAAAACCCAAGT	56
7	69	172	50	CTTTGQAGCTAACCTTTTACG	74	CAAGAGGATTTCTATTCTGAA	94
7A	138	242	51	GCTGATAACATGTCTGCTTTA	47	ATTAGGGCTGGTGGTGGTAGC	12
8	111	213	59	TTGCCTTTGCTGTGCTTGAG	27	AGTCTCCTGTGGGCTGCTGAG	33
9	99	231	54	TTCTGACTCCCTGTGTGAACA	29	CTGCCAGAATAGACATCAAGT	61
10	78	198	56	TGCTGTGGCGTTTGTCAC	55	TGAAGCTGAAAAGGCACT	29
11	117	188	59	CTGCGGCCTTTCCTTTTC	2	GGCTACCACCTTGAGGGT	33
12	67	164	55	GTCTGCTGTCCTCGGTGCTAA	19	GGGGTGCTGTTTGATTTTCTT	36
13	88	278	58 + DMSO	GGCCAGAGTGCACCACACAAAG	113	CCCCAGCACAATAAAACCTTAG	35
14	94	316	53	ACTTAAATGCCAAAGCACTGC	147	GAATGAAACCAGAAAACAAAC	33
15	539	514	59	CGTTCTGTTCCCTGAGGTGGC	37	CTCCGGTTCTTGTTCCTAAGC	288 to TGA

Table 4 Polymorphisms detected in human *GRB10*

GRB10 *intron/exon*	*Nucleotide change*	*Amino acid change*	*Domain*	*Enzyme site change*	*HSCR patients*			*Controls*			χ^2 (P)
					+/+	+/−	−/−	+/+	+/−	−/−	
intron 1	ATA→GTC	15 bp 3′ of exon 1	unique to GRB-IR	*Tai*I	102	1	6	275	25	15	1.33 *(0.249)*
exon 3	CCG→CCA	P105P	unique to GBR-IR	*Ava*I *Sty*I	38	26	60	16	53	38	0.045 *(0.832)*
exon 6	TAC→TAT	Y249Y	GM	–	75	8	0	74	8	0	0.857 *(0.355)*
exon 6	CCC→CCA	P258P	GM	*Sty*I	78	5	0	74	8	0	0.346 *(0.556)*
intron 6	G→A	3 bp 3′ of exon 6	GM	*Hph*I *Ple*I, *Hinf*I	79	4	0	74	8	0	0.760 *(0.383)*
intron 11	GTGGG→ ATGGA	18 bp 5′ of exon 12	GM	–	72	6	0	80	3	0	0.552 *(0.457)*

GM = Grb and Mig (see text). '+' refers to previously published alleles in *GRB10* cDNA (Frantz *et al*., 1997; Liu and Roth, 1995; O'Neill *et al*., 1996), '–' refers to alleles not previously described

is described in Table 1 and contains the GT-AG consensus sequences for eukaryotic donor and acceptor splice sites (Senapathy *et al*., 1990).

Several factors might explain why *GRB10* was not found to predispose to HSCR in our study. First, although there is little doubt that *Grb10* and *Ret* interact in the mouse, this may not be the case in the human. Also, it is not clear that this interaction is essential *in vivo*; in the absence of targeted *Grb10* mutations in mice, it is difficult to gauge whether *Grb10* is truly unique in its function, or is merely a redundant member of a large family of SH2 adapter proteins. Closely-related family members Grb7 and Grb14 also contain proline-rich, GM, PH and SH2 domains. Moreover, Ret appears to be one of several signaling molecules that exhibits high affinity for Grb10. Both the insulin receptor (IR) and the type I insulin-like growth factor receptor (IGF-IR) have been shown to interact directly with Grb10 (reviewed in (Morrione *et al*., 1997). Also, recent reports have shown that human GRB10 isoforms are differentially expressed in insulin target cells such as skeletal muscle, liver and adipocytes (*Dong et al*., 1997). Thus, GRB10 may be more important in mediating insulin signaling than in enteric neurogenesis. Lastly, although we were able to screen all of the *GRB10*/*GRB-IR* exons in patients and controls, we did not screen the introns or the upstream and downstream untranslated sequences. In addition, given that exon 7A was found to reside in intron 7, it is not unlikely that additional exons may exist within other introns. Indeed, there are now four known isoforms in the human (Dong *et al*., 1997). Nevertheless, linkage analysis of HSCR families using a highly polymorphic microsatellite marker within the gene suggests that *GRB10* mutations cannot account for a substantial fraction of familial HSCR.

To date, all reported HSCR susceptibility genes are members of the RET signaling pathway (*RET, GDNF*), the G protein-coupled receptor (GPCR) endothelin-B pathway (*EDNRB, EDN3*), or the SRY-like HMG-box family of transcription factors (*SOX10*; Pingault *et al*., 1998; Southard-Smith *et al*., 1998). We believe that the presence of both SH2 and PH domains in the Grb10 family of proteins suggest a possible model for uniting the RET and GPCR pathways. There is now evidence that human GRB10 is a common target for kinases existing in both the MAP kinase and phosphatidylinositol 3-kinase signal transduction pathways (Dong *et al*., 1997). These data, as well as the importance of SH2 domains in RTK signaling and PH domains' possible role in G protein-coupled receptor-mediated signaling, suggest that GRB10 family members might serve as a conduit for transducing extracellular neuroenteric signals to the nucleus and perhaps, SOX10 or other related transcription factors therein.

Acknowledgements
We are extremely grateful to the families who make all of our studies possible, to Dr Akhilesh Pandey for reagents and helpful discussion, and to Nydia Bringht-Twumasi for expert technical assistance. This research was partially supported by a grant from the National Institutes of Child Health and Development, National Institutes of Health (HD-28088).

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