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Apolipoprotein B is a new target of the GDNF/RET and ET-3/EDNRB signalling pathways

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Abstract

Background *GDNF/RET and Endothelin-3 (ET-3)/EDNRB regulate survival, differentiation, migration, and proliferation of neural crest-derived cells. Although several RET and EDNRB signalling mediators have been characterized, most of the genes targeted by these two pathways are still largely unknown. We focused our study on apolipoprotein B (APOB) as a novel target gene of the RET and EDNRB pathways, based on previous data obtained using a Caenorhabditis elegans strain mutant for the homologue of mammalian ECE1.* ***Methods*** *Molecular and cellular studies of Apob were performed in the murine Neuro2a cells, an in vitro model for studying neural crest-derived cell development, along with a mouse knock-in for the Hirschsprung-associated mutation* Ret^{C620R}*. Silencing for Apob and Ret has been performed via shRNA.* ***Key Results*** *GDNF/RET and ET-3/EDNRB cooperated in inducing neuronal differentiation resulting in Apob activation in Neuro2a cell line. Apob expression was downregulated in mouse embryos homozygous for the* Ret^{C620R} *mutation and presenting a severe Hirschsprung phenotype. Ret silencing prevented Apob expression increase. MAPK P38 kinase activation evoked Apob expression via GDNF/RET signalling in Neuro2a cells. A p53-dependent repressor element in Apob promoter resulted in a reduced Apob expression. Silencing of Apob reduced HuD protein expression.* ***Conclusions & Inferences*** *Apob is a novel downstream target of the RET/EDNRB pathways with a role in neuronal survival and maintenance, as indicated by its effect on HuD expression. Our data provide a conceptual framework to investigate and establish the role of APOB gene in severe gut dysmotility.*

Keywords *apolipoprotein B, enteric nervous system, Hirschsprung's disease, Neuro2a cell line,* Ret^{C620R}*.*

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INTRODUCTION

An intact and functioning enteric nervous system (ENS) is required for life. The segmental absence of the ENS, which occurs either as a congenital [Hirschsprung's disease (HSCR)] or acquired defect,[1] causes a severe impairment of gastrointestinal transit. Hirschsprung's disease is characterized by the absence of enteric ganglia in the intestinal tract, with an incidence of approximately 1/5000 births affecting more men than women (4 : 1 ratio).[2,3]

RET is the main gene involved in HSCR: the majority of patients harbor either heterozygous mutations in the coding region or a heterozygous or homozygous hypomorphic allele in a conserved enhancer sequence in intron 1.[4,5] Mutations in other genes encoding proteins involved in either RET or Endothelin receptor type-B (EDNRB) signalling pathways are found in a small percentage of HSCR patients.[2,3]

RET codes for a tyrosine kinase transmembrane receptor, with two alternative isoforms at the

C-terminal recruiting different signalling factors.[6] Activation of RET occurs upon self-dimerization, induced by the binding of glial cell line-derived neurotrophic factor (GDNF) family ligand to glycosylphosphatidylinositol-anchored co-receptor GDNF family receptor α and resulting in the activation of three main kinases, Akt, ERK, and MAPK P38.[4] RET activation regulates ENS normal development by modulating survival, differentiation, migration, and proliferation of enteric neural crest-derived cells.

EDNRB is a G-coupled receptor that binds any of three endothelins (ET-1, -2, -3),[7] produced as prepro-endothelins and proteolytically activated by Endothelin-converting Enzyme-1 (ECE-1). The crosstalk between GDNF/RET and ET-3/EDNRB pathways in coordinating ENS development has been demonstrated in *in vitro* and *in vivo* studies.[8–11] Nevertheless, the molecular bases of this crosstalk remain to be elucidated.

This study was designed to investigate apolipoprotein B (APOB) as a novel target of the crossregulation between RET and EDNRB pathways, as emerged from previous studies using the *Caenorhabditis elegans* strains mutant for the mammalian *ECE1* homologue.[12] *APOB* is expressed in two isoforms, APOB100 and APOB48, the former being synthesized in the liver as an essential component of lipoproteins, and the latter being generated by RNA editing in the gut for chylomicron production.[13] *APOB* mutations cause familial hypobetalipoproteinemia[14] and familial defective apolipoprotein B-100.[15]

A correlation between APOB and the genes involved in ENS development, such as EDNRB and RET, has not been reported so far. However, *Apob* knockout mice showed severe neural tube defects suggesting a role for APOB during embryogenesis and in particular with regard to neural crest development.[16] We therefore focused on the Neuro2a murine cell line, an *in vitro* model for studying neural crest development and neuronal differentiation,[17,18] along with a mouse knock-in for the mutation Ret^{C620R} associated to HSCR[19] to investigate the role of Ret and Ednrb signalling pathways in *Apob* expression and to identify the molecular partners involved in this process. We demonstrated that *Apob* expression is actually activated by GDNF/Ret via MAPK P38 and modulated by ET-3/Ednrb. Furthermore, we identified p53 as a transcription factor involved in the regulation of *Apob* expression. Taken together our data provide a basis to investigate the role of *APOB* in the clinical setting specifically focusing on severe gut dysmotility disorders.

MATERIALS AND METHODS

Cell cultures and growth factor treatments

Murine Neuro2a cells were grown in DMEM, supplemented with 10% fetal bovine serum (FBS), 2 mm L-glutamine, at 37 °C in a 5% CO_2 humidified atmosphere. SK–N–MC and SK–N–BE were grown in DMEM/F12 1/1 v/v and RPMI, respectively, supplemented with 2 mm L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10% FBS at 37 °C in a 5% CO_2 humidified atmosphere.

After 16 h serum starvation, Neuro2a cells were treated with 50 ng mL^{-1} GDNF (R&D Systems, Minneapolis, MN, USA) and 300 nmol L^{-1} ET-3 (Sigma-Aldrich, St. Louis, MO, USA), separated and in combination for different times. To test RET-activated signalling pathways, Neuro2a cells were shifted to a serum free medium in the presence of kinase inhibitors before GDNF treatment, as follows: TCN (Triciribine V) (Akt inhibitor) 10 μm 24 h; SB202190 (MAPK38 inhibitor) 20 μm 24 h; PD98059 (ERK inhibitor) 40 μm 24 h. To test Neuro2a cell growth and differentiation upon GDNF and/or ET-3 treatment, Neuro2a cells were seeded on PolyD lysine (Sigma-Aldrich) precoated 24-well plates to a final density of 2500 or 5000 cells. The following day the medium was replaced with DMEM supplemented with 2 mm L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.5 μg mL^{-1} DMPH4, 2.5 μg mL^{-1} L-GSH, 50 μg mL^{-1} L-ascorbic acid, and N2 Supplement (synthetic medium) and cells were treated with GDNF (10 ng mL^{-1}) and ET-3 (300 nmol L^{-1}) separately and in combination. Triplicate wells were studied for each condition, including three wells in which cells were grown in the synthetic medium without the addition of any growth factors (control cultures). For time course observations, cells were plated in multiple 24-wells plates, fixed in methanol (–20 °C) at a particular time point (every 24 h) for up to 5 days. Half of the medium was replaced once every 2 days. At the end of the treatment, cell growth was evaluated by counting cells present in each well using phase-contrast microscopy with the help of a grid placed in the ocular and lines drawn on a transparent film attached to the 24-multiwell plate in such a way as to obtain distinct fields to be evaluated separately. Cell differentiation was evaluated by a detailed analysis of cell morphology by phase-contrast microscopy. Cells were classified according to the presence and shape of neurites in six groups: undifferentiated, round-shaped cells; cells with one neurite (monopolar), cells with two opposite neurites (bipolar); cells with two opposite and branched neurites (branched-bipolar), cells with many neurites (star cells); cells with one prevalent branched neurite (branched) (Figure S1).

Mice knock-in Ret^{C620R}

Ret^{C620R} mice were grown at Charles River animal facility (Lecco, Italy). Experimental procedures were approved by the ethical committee for animal experimentation of the University of Bologna, in accordance with European legislation (European Communities Council directive of November 24, 1986, 86/609/EEC).

Western blotting

Whole-cell lysates were obtained by scraping cells directly in IP Buffer 1X containing deoxycholate (Sigma-Aldrich) and a cocktail of protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitors (Sigma-Aldrich) on ice for 15 min.

Cells were collected and lysed through an insulin syringe. Protein concentration was evaluated with the DC protein concentration assay Kit (Biorad, Hercules, CA, USA). Proteins were denatured by heating at 95 °C for 5 min in Laemli buffer, separated by SDS gel electrophoresis, transferred onto nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, UK), and subjected to Western blotting. Bands were visualized using the ECL method (GE Healthcare).

Primary antibodies used were as follows: anti-PAN-RET 1 : 200, anti-phospho-Akt 1 : 1000, anti-total Akt 1 : 1000, anti-phospho-Erk 1 : 1.000, anti-total Erk 1 : 1000 (Santa Cruz, Biotechnology Inc., Santa Cruz, CA, USA), anti-phospho MAPK P38 kinase 1 : 1000, anti-MAPK P38 kinase 1 : 1000 (Cell Signaling, Danvers, MA, USA), anti-HuD 1 : 100 (Invitrogen, Carlsbad, CA, USA), anti-γ-tubulin 1 : 10 000, anti-vinculin 1 : 30 000 (Sigma-Aldrich).

Ret and *Apob* silencing via shRNA

Neuro2a cells (5×10^5) were transfected either with the pRS-shRNA vectors (OriGene, Rockville, MD, USA) (specific for mouse *Ret,* TR1504590, and *Apob* silencing, TI562711), or the empty vector, or the scramble vector (control) using lipofectamine (Invitrogen). After 24 h, cells were divided into the number of wells necessary for GDNF stimulation. The next day, cells were shifted to a serum-free medium for 16 h. GDNF treatment and protein extraction were performed as previously described. Lack of Ret expression was verified via Western blotting using the PAN-RET antibody (1 : 200). RNA was extracted and *Apob* silencing was verified by real-time quantitative RT-PCR as described below.

Immunocytochemisty

Neuro2a cells were fixed in 4% PFA, washed in PBS and incubated in a 0.3% H_2O_2/Methanol solution for 15 min, then incubated for 60 min at 4 °C in blocking solution (1% BSA/PBS) and washed three times for 30 min in PBS. Two primary anti-RET antibodies were used obtaining similar results: anti-Ret antibody (R&D Systems) and anti-PAN-RET (Santa Cruz). Cells were incubated with primary antibody at a 1 : 200 dilution for 2 h, washed in PBS and incubated for 1 h with biotinylated secondary antibody (Santa Cruz) at a 1 : 200 dilution. Slides were washed three times for 30 min in PBS. Staining was performed using the Vectastain Elite ABC kit (Vector, Burlingame, CA, USA) and the DAB substrate kit for peroxidase (Vector). SK–N–MC and SK–N–BE neuroblastoma cell lines were used as negative and positive control, respectively.[20,21]

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded adult human colon tissues (patients routinely operated for uncomplicated colon cancers commonly used as controls for immunohistochemical experiments) according to protocols validated in our laboratory.[22] Briefly, tissue sections were treated to remove paraffin embedding by three sequential washes in xylene and graded ethanol. Antigen unmasking was performed by heating sections at 95 °C in 10 mm Sodium citrate buffer, pH 6.0, for 10 min and subsequent cooling at room temperature for 30 min. To reduce endogenous peroxidases, tissue sections were treated with an ad hoc blocking kit (GeneTex Inc., Irvine, CA, USA) and then incubated for 16 h at 4 °C with primary antibodies, i.e. anti-human APOB diluted 1 : 100 (ABCAM, Cambridge, UK); anti-human NSE diluted 1 : 250 (Santa Cruz). Fluorescent secondary antibodies were diluted in Triton-X (1 : 300) and incubated for 60 min at room temperature, before subsequent dehydration and visualization under a LEICA DMLB fluorescent microscope.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted using the Trizol method and was reverse transcribed using Superscript III kit and random hexamers (Invitrogen). Primers for *Apob*, *Actb* were designed with Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and RTPrimerDB (http://medgen.ugent.be/rtprimerdb/). Quantitative real-time polymerase chain reaction was performed with SYBRGreen, 0.5 μm primers, in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster city, CA, USA). All target genes were normalized with the corresponding endogenous control using the ΔC_t comparative method; *Apob* expression levels were normalized to *beta-actin* (*Actb*).

Expression of *Ret*, *Gfra1* and *Ednrb* in Neuro2a cells was assessed by RT-PCR using mouse brain RNA as positive control. All primers used in the present work are available on request.

Expression of *Apob* in mouse embryos Ret^{C620R}

Ret^{C620R}/Ret^{C620R}, $+/Ret^{C620R}$ and wild-type embryos were removed by Caesarian section from pregnant carrier mothers at 16 days post coitum (d.p.c.) and genotyped as described previously.[19] Tissues were preserved in RNAlater (QIAGEN, Valencia, CA, USA) and total RNA was extracted using the Trizol method. qRT-PCR was performed as described above.

Mouse *Apob* promoter analysis

Mouse *Apob* upstream region was amplified using primer 5′-GGGCTCGAGGGCTTATGGCCTCTGTATGC-3′ inserting an *XhoI* restriction site at the 5′ end, and primer 5′-GGGAAGCTTCCTGCTGCTTGCTCTACAGG-3′ inserting a *HindIII* restriction site at the 3′ at the following condition: 60 ng mouse genomic DNA, 0.4 μm primers, 0.4 mm dNTPs, 2.5 mm $MgCl_2$, DMSO 5%, 0.2 μL Platinum Taq in a final volume of 50 μL. The PCR amplification was performed as follows: 10 min 94 °C, 1 min at 94 °C, 30 s at 62 °C, 1 min at 72 °C, 46 cycles. The PCR product was inserted in the pGL3 basic vector and different deleted clones were generated with the Erase-a-base kit (Promega, Madison, WI, USA). The mutated promoter construct at −986 bp was generated with the QuickChange site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA). 400 ng of each construct was independently co-transfected with 400 ng of the control vector containing the renilla luciferase reporter into 2×10^5 Neuro2a cells using lipofectamine in duplicate. 24 h after transfection, cells were shifted to a serum free medium for 16 h, treated for 1 h with GNDF (50 ng mL^{-1}) and lysed in PLB 1X (Passive Lysis Buffer) for the Dual-Luciferase Reporters assay (Promega). Firefly luciferase values were normalized to renilla luciferase values.

Electromobility shift assay (EMSA) on mouse *Apob* -986bp region

10^7 Neuro2a cells were rinsed in cold PBS-Na_3VO_4 0.2 mm, pelleted by centrifugation at 1600 ***g*** and lysed in 400 μL of Buffer A (10 mm HEPES pH 7.9, 10 mm KCl, 0.1 mm EDTA, 1 mm DTT, 1 mm Na_3VO_4 and a protease inhibitors cocktail) for 15 min in ice. A quantity of 25 μL of 10% NP-40 was added and the samples were centrifuged at 16 000 ***g*** 30 s. The nuclear pellets were resuspended in 50 μL Buffer C (20 mm HEPES pH 7.9, 0.4 mm KCl, 0.1 mm EDTA, 0.1 mm EGTA, 1 mm DTT, 1 mm

Na_3VO_4 and a protease inhibitors cocktail), incubated on ice for 45 min, and centrifuged at 16 000 ***g*** 10 min at 4 °C. Supernatants were subjected to EMSA or stored at −80 °C until use. EMSA shift was performed with the LightShift Chemiluminescent EMSA Kit (Thermo Scientific Inc., Pittsburg, PA, USA). A quantity of 10 μg of nuclear extracts, 20 fmol of 5′ biotin-ATGCATTTCT-ATGCGTGCATGCCCATGAA probe, 50 ng of poly(dI), 2.5% glycerol, 0.05% NP-40 and 5 mm $MgCl_2$, were incubated at room temperature for 20 min in 20 μL. For the supershift assay, nuclear extracts were pre-incubated with anti-p53 mouse monoclonal antibody (0.6 μg; Santa Cruz) on ice for 20 min prior to probe addition. For competition assay, 100-fold excess unlabeled double-stranded oligonucleotides were incubated. Bound complexes were separated on 4% polyacrylamide gels, blotted onto nylon membrane and visualized according to the LightShift Chemiluminescent EMSA Kit.

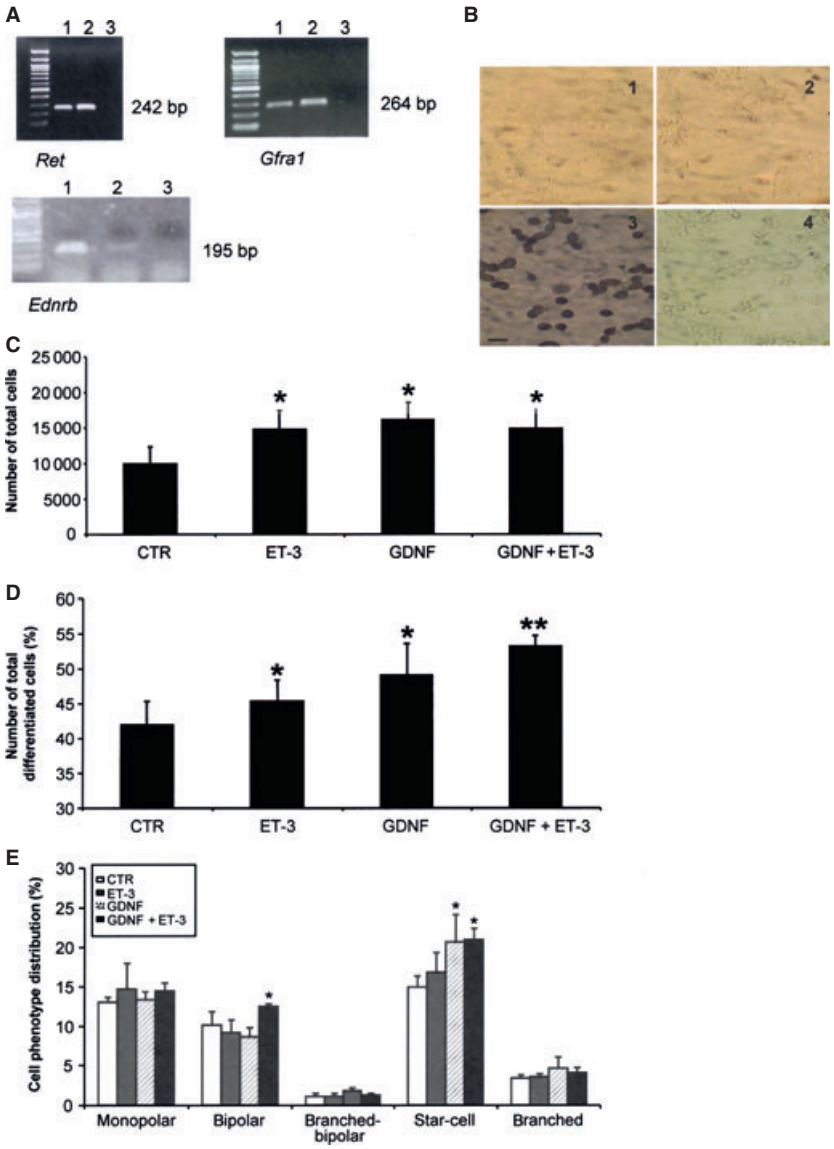

Figure 1 Effect of GDNF and ET-3 treatment on Neuro2a cell number and differentiation. (A) Expression of *Ret*, *Gfra1*, and *Ednrb* in mouse brain (1) and Neuro2a cells (2); (3) negative PCR controls. (B) Ret protein expression in Neuro2a cells. Immunolabeling with anti-Ret primary antibody of SK-N-MC that do not express Ret (1) and Neuro2a cell lines (3). (2, 4) Negative controls of NK-N-MC and Neuro2a cells, respectively. (C) Neuro2a cell numbers in cultures plated at moderate-high cell-plating densities at the end of a 4 days treatment with GDNF (10 ng mL^{-1}) and ET-3 (300 nm) separated and in combination. Data represent means ± SE (D) Total differentiated Neuro2a cells in cultures plated at low cell-plating densities (2500 cells per well) at the end of a 4 days treatment with GDNF (10 ng mL^{-1}) and ET-3 (300 nmol L^{-1}) separated and in combination. (E) Cell-phenotype distribution in the analyzed cells. Data represent percentage of differentiated cells (±SE) observed in samples of 500 cells per replica-well for treatment conditions. Cells were classified in one of the six different cell phenotypes: monopolar, bipolar, branched-bipolar, star, and branched cells.

Statistical analysis

Statistical analysis was performed using Statgraphics Plus 5.1. software (Statistical Graphics Corp., Warrenton, VA, USA). The Student's *t*-test was used to compare two groups, ANOVA and chi-square test were used for multiple comparisons. A value of $P < 0.05$ was considered significant. Data are expressed as means ± SE.

RESULTS

ET-3/Ednrb signalling modulates GDNF/Ret-related cell growth and differentiation and *Apob* expression

We tested the existence of a crosstalk between Ednrb and Ret in Neuro2a cells and established whether or not these molecular pathways modulated *Apob* expression. The expression of *Ret*, *Gdnfra,* and *Ednrb* in Neuro2a cells has been verified by RT-PCR, whereas Ret protein has been detected using immunocytochemistry (Fig. 1A and B).

At a density of 5000 cells per well, the administration of GDNF exerted a more potent stimulatory effect on Neuro2a cell proliferation compared to ET-3 (ANOVA, $P < 0.05$; chi-squared test, $P < 0.0001$). As expected, the concomitant administration of both ligands resulted in a lower Neuro2a cell proliferation due to molecular interference of the two pathways (Fig. 1C). At a density of 2500 cells per well, Neuro2a cells treated with GDNF and ET-3 for 4 days showed the most evident differentiation effect. Differentiated Neuro2a cells were classified into six different phenotypes (Figure S1). Differentiated cells were observed as a result of Neuro2a treatment with either GDNF or ET-3 (chi-squared test, $P = 8.04 \times e^{-05}$ and $P = 0.04$, respectively); the combination of the two ligands induced a stronger effect on the differentiation process (ANOVA, $P < 0.00001$; chi-squared test, $P = 4.6 \times e^{-10}$) (Fig. 1D). Cell-phenotype analysis indicated that after the combined GDNF and ET-3 treatment, cells were strongly induced to differentiate in two main phenotypes (i.e. bipolar and star-like) (chi-squared test, $P = 0.034$ and $P = 9.2 \times e^{-06}$, respectively). GDNF alone induced cells to differentiate to the star-like phenotype (chi-squared test, $P = 2.33 \times e^{-05}$) (Fig. 1E).

To determine whether or not *Apob* is modulated by GDNF/ET-3, we treated cells with the two ligands separately and in combination as reported in Fig. 2. We measured *Apob* mRNA levels through the qRT-PCR and data were normalized using the *β*-actin gene as endogenous control. GDNF induced an early strong increase in *Apob* expression after 30 min of stimulation (Fig. 2A), whereas, compared to GDNF, the treatment with ET-3 alone gave a less prominent *Apob* increment (Fig. 2B). The combination of GDNF and ET-3 led to an increase, although delayed in time, in *Apob* expression (Fig. 2C).

Apob expression is activated by GDNF/RET signalling pathway

To confirm that the increase in *Apob* expression was an exclusive effect of Ret activation, we silenced *Ret* by transient transfection with a *Ret*-specific short hairpin RNA. Transfected Neuro2a cells were treated with GDNF (50 ng mL^{-1}) at different times (30 min, 1 h, and 3 h), and protein and RNA were extracted. Neuro2a cells silenced for *Ret* did not show any *Apob* increase, confirming that the GDNF/Ret pathway activated *Apob* in a specific manner (Fig. 3A, upper panel). Western blotting confirmed the absence of Ret in the silenced cells (Fig. 3A, lower panel). Transfections with the empty or scramble vector did not abolish Ret-induced *Apob* increase (data not shown).

The inhibition of the GDNF/Ret-dependent MAPK P38 kinase with SB202190 completely abolished *Apob*

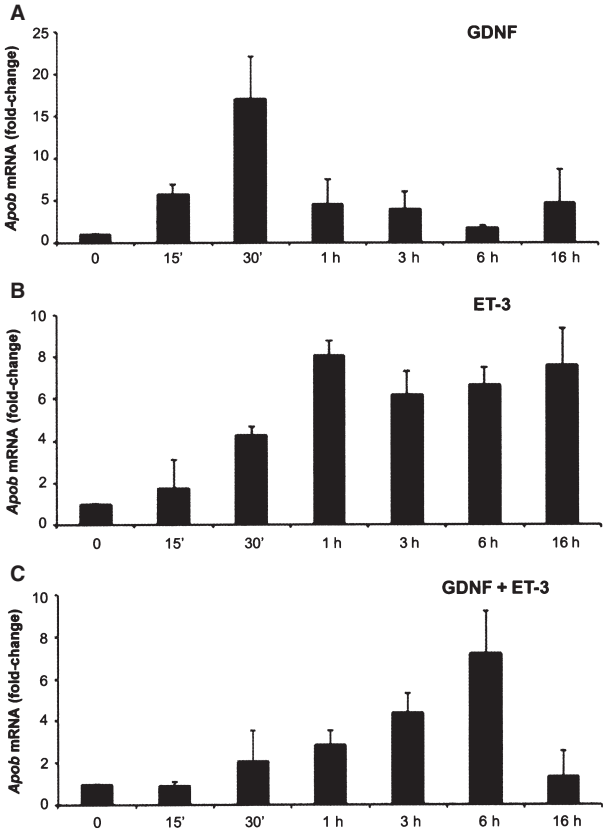

Figure 2 Effect of GDNF and ET-3 treatment on *Apob* expression. qRT-PCR of *Apob* in Neuro2a upon stimulation with GDNF (A), ET-3 (B) and GDNF and ET-3 in combination (C). Data represent the mean (±SE) from three independent experiments.

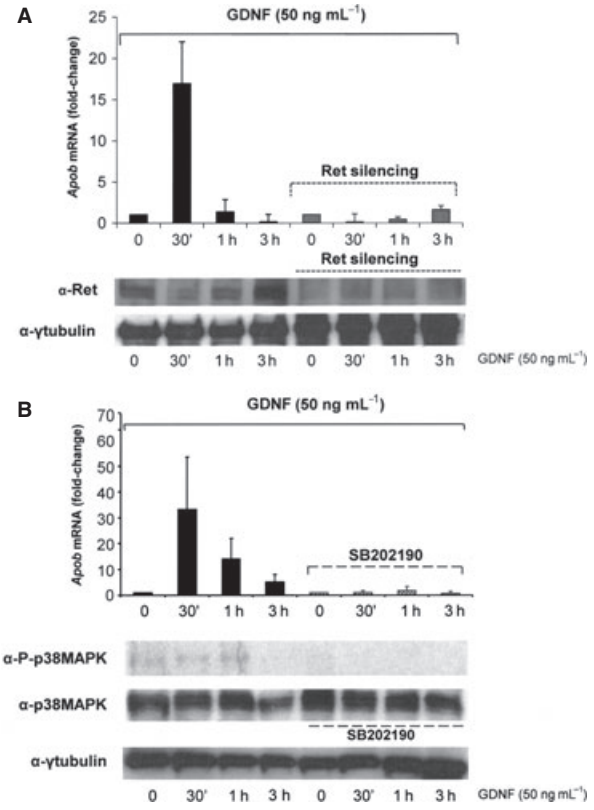

Figure 3 Specific activation of *Apob* transcription by RET/GDNF via MAPK P38 kinase. (A) Ret silencing abolished *Apob* increased expression in Neuro2a. Upper panel: qRT-PCR for *Apob* expression in GDNF-treated Neuro2a in GDNF-treated Neuro2a with *Ret*-silenced (dashed lines). The data represent the mean (error bars) from three independent experiments. *Apob* mRNA levels are normalized to A*ctb*. Lower panel: Western blot analysis for Ret protein in serum starved GDNF-treated Neuro2a (first four lanes) and in serum-starved Neuro2a silenced for Ret and treated with GDNF (last 4 lanes). (B) Activation of *Apob* transcription by RET/GDNF via MAPK P38 kinase. Upper panel: qRT-PCR for *Apob* expression in GDNF-treated Neuro2a in absence and presence of SB202190 (dashed lines). Lower panel: Western blot analysis of GDNF-treated Neuro2a in absence or presence (last four lanes) of SB202190.

upregulation (Fig. 3B), whereas Akt and Erk kinase inhibitors did not modify its expression (Figure S2). Therefore, MAPK P38 kinase activation by Ret is responsible for the *Apob* increase.

Apob silencing reduces HuD expression

These experiments were aimed to understand whether or not *Apob* activation contributes to ENS development. In this line, we assessed HuD protein changes as an index of neuronal differentiation[23] following GDNF treatment of Neuro2a cells transiently silenced for *Apob*. As shown in Fig. 4A and B, in GDNF-treated Neuro2a cells transfected with the scramble vector (control) the *Apob*-increased expression was associated with HuD protein increase, an effect prevented by *Apob* silencing.

Expression analysis of *Apob* in HSCR mouse models

To establish whether or not GDNF/Ret-induced *Apob* expression played a role on HSCR pathogenesis, we evaluated *Apob* expression in a mouse knock-in model for the Ret^{C620R} mutation. $Ret^{C620R/C620R}$ mice show severe agangliogenesis, kidney agenesis, and die after birth, whereas the heterozygous mice show a normal organ development.[19] We tested the expression of *Apob* in wild-type, heterozygous, and homozygous mouse embryos at day 16 via qRT-PCR (Fig. 5). Compared to wild-type, *Apob* expression was lower in homozygous mice (Student's *t*-test, $P = 0.0372$), whereas there was a striking increase in expression in heterozygous mice (Student's *t*-test, $P = 0.0052$) as a result of a functioning Ret.[19]

APOB immunolabeling in human colonic tissues

Apolipoprotein B immunoreactivity was identified in cells (with morphological features of immunocytes) of the *lamina propria* of the adult normal human colon. In contrast, no APOB immunolabeling was detected in enteric neuronal cell bodies or nerve fibers, epithelial cells, or other cell targets of human colon tissue sections (Fig. 6).

Identification of GDNF/Ret-dependent *cis* regulatory regions in mouse *Apob* promoter

To determine the GNDF/Ret-dependent regulatory elements in the mouse *Apob* genomic region, we first predicted the promoter region *in silico* using two different softwares: Gene2promoter (http://www.genomatix.de) and PromoterScan. Both programs indicated the region between bp 4983663–4984504 of contig NT_039548 from mouse chromosome 12 as the predicted promoter. We cloned the region upstream of the mouse *Apob* gene, including the predicted *Apob* promoter, from genomic DNA and inserted it into the pGL3 vector containing the firefly luciferase gene as reporter. Constructs with progressive deletions of the cloned region were obtained by sequential digestion, and the plasmids were characterized by sequencing (Fig. 7A).

Each construct was co-transfected into Neuro2a cells with the control vector containing the renilla luciferase gene under the control of the SV40 promoter. One day after transfection, cells were serum starved for 16 h

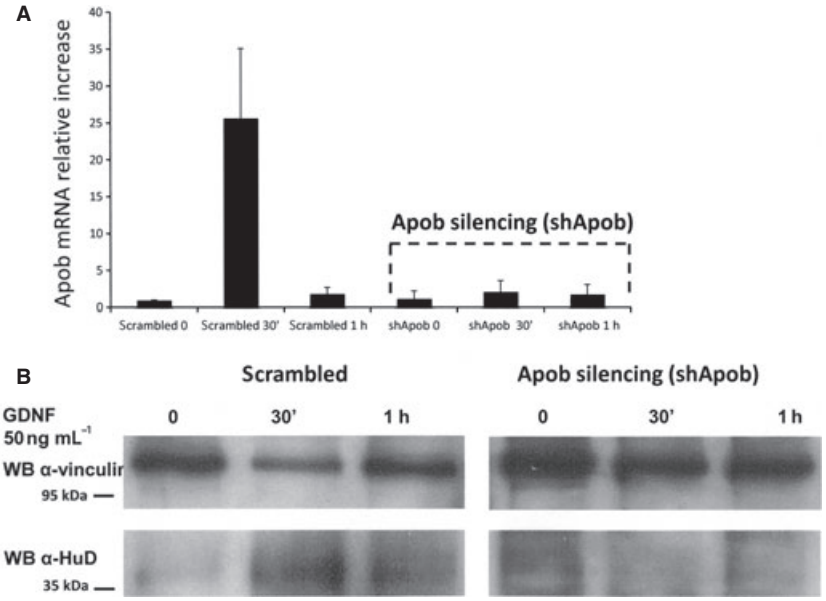

Figure 4 *Apob* silencing reduces HuD expression in GDNF-treated Neuro2a cells. (A) Relative *Apob* expression measured via real-time qRT-PCR in cells transfected with the scramble vector or with the shRNA vector for mouse *Apob* silencing (shApo) and treated with GDNF at different times. (B) HuD protein levels in Neuro2a cells transfected with scramble vector or with the shRNA vector for mouse *Apob* silencing (shApo) and treated with GDNF at different times (lower panel). Upper panel: Western blot for vinculin.

and GNDF stimulation was performed before cell lysis for the luciferase assay. No luciferase activity was found in absence of GDNF (Figure S3). All data were compared to the activity of the 900-bp construct, as this was the minimal promoter region defined by Gene2promoter and PromoterScan. The 1000-bp construct showed a significantly reduced activity compared to the 900-bp construct ($P = 0.00020$, Student's *t*-test), suggesting that the 100 bp extra-region contains repressor elements of *Apob* expression (Fig. 7B). to identify the *cis* regulatory elements responsible for *Apob* GDNF/Ret-dependent transcription regulation, we performed an *in silico* analysis of this region using MatInspector (http://www.genomatix.de). One site with a high matrix score (thus indicating a real binding site for transcription factors) was identified and represented a binding site for p53 (−986 bp site, matrix score = 0.934) (Fig. 7C). Site-directed mutagenesis of the 1000-bp construct generated a deletion of 2 bp in the p53-binding element (Fig. 7D; upper panel). Activities of wild-type and mutated 1000-bp constructs were compared to the activity of the 900-bp construct via the luciferase assay. The mutated 1000-bp construct showed a comparable activity with the 900-bp construct ($P = 0.9457$), whereas the original 1000-bp construct activity was reduced ($P \leq 0.0001$) (Fig. 7D; lower panel). These data indicate that the p53-binding element acts as a repressor of *Apob* expression.

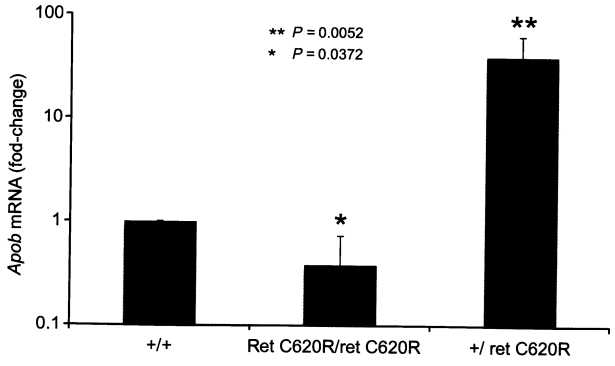

Figure 5 *Apob* expression in mouse embryos heterozygous and homozygous for Ret C620R (embryonic day 16.5) via qRT-PCR analysis. Data are expressed as means ± SE.

We performed an EMSA to verify that p53 actually binds to the region. As shown in Fig. 7E, we first verified that the binding of the Neuro2a nuclear extract to the biotin-labelled p53-putative binding site was specific, as it disappeared in the presence of the unlabelled probe (Fig. 7E, third lane). Next, we were able to detect a supershift when the nuclear extract was pre-incubated with an anti-p53 antibody (Fig. 7E), confirming the binding of p53 to the region.

DISCUSSION

RET and EDNRB are critical players during the development of the ENS, as mutations in genes encoding members of either RET or EDNRB pathways lead to a

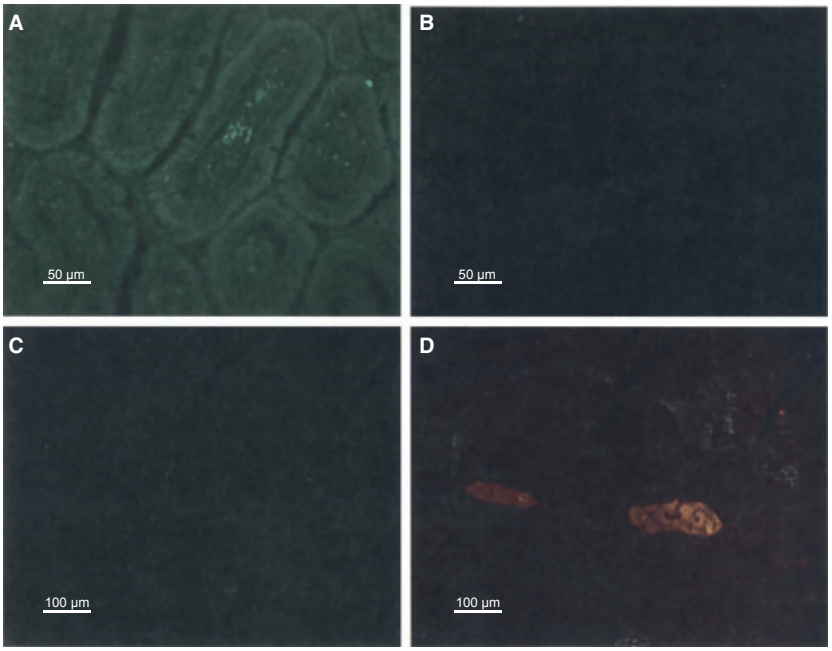

Figure 6 Representative examples showing APOB and NSE immunoreactivity in the adult human control colon. (A) Illustrates an intense APOB labeling in cells of lamina propria with features of immunocytes. (B) Shows the lack of APOB immunoreactivity as a control obtained by pre-incubating the primary antibody (1 : 100) with APOB peptide (10^{-5} m). (C) Shows the lack of APOB immunoreactivity in the myenteric plexus which was clearly identified by NSE labeling (D). Mouse anti-goat and anti-rabbit secondary antibodies conjugated either with fluorescein isothiocyanate (FITC) or tetramethylrhodamine-isothiocyanate (TRITC) were used at 1 : 300 dilution each. Calibration bars: 50 and 100 μm, respectively.

HSCR phenotype. Although several studies have already identified genes that are modulated at the end of each signalling cascade and novel downstream genes activated by GDNF/RET have been identified,[24,25] genes modulated by the crosstalk between these two pathways are still matter of investigation.

We have previously identified a vitellogenin (*vit-3*), corresponding to human APOB, as a differentially expressed gene in *C. elegans* strains null for the worm homologue of mammalian *ECE1*.[12,26] Several studies have shown the role of APOB in lipid transport and chylomicron production in the gut.[13] Nevertheless, *APOB* mutations have been found in hypobetalipoproteinemia and defective apolipoprotein B-100[14,15] and APOB levels have been mainly associated to the risk of chronic heart disease[27] and age-related macular degeneration.[28] However, a possible correlation between APOB and neuronal function/development arises from studies in *Apob* homozygous null mice, which displayed neural tube defects and died *in utero*.[16] Thus, the present study was undertaken to investigate whether or not *Apob* expression has a role in the proliferation/differentiation effects induced by Ret and Ednrb signalling pathways in the Neuro2a cell line, an *in vitro* model for the study of neuronal development and differentiation.[17,18] We have demonstrated that GDNF was a stronger inducer of cell proliferation compared to ET-3. When combined together, ET-3 partially inhibited the proliferative effect of GDNF, in accordance with previous results in enteric neural crest-derived cells committed to neuronal lineage.[10] In our cell model, i.e. the Neuro2a cell line, the combination of GDNF and ET-3 induced cells to differentiate into bipolar and star-like phenotypes. Furthermore, GDNF promoted an early increase in *Apob* expression, which was delayed in combination with ET-3. These data paralleled the effect of GDNF/ET-3 on cell proliferation/differentiation described above, suggesting that the GDNF-mediated early *Apob* increase may be related to the proliferative effect on neural-crest-derived cells.

We demonstrated that *Apob*-increased expression was a specific Ret-induced effect due to GDNF stimulation, because *Ret* silencing abolished *Apob* upregulation. This increase was dependent on the MAPK P38 kinase pathway, as MAPK P38 inhibition blocks Ret-induced *Apob* activation. MAPK P38 kinase plays a key role in the balance between cell proliferation and cell death induced by different stimuli.[29] To gain further information on the downstream signals activated by Ret via MAPK P38, we evaluated the potential regulatory factors that mediated *Apob* increase.

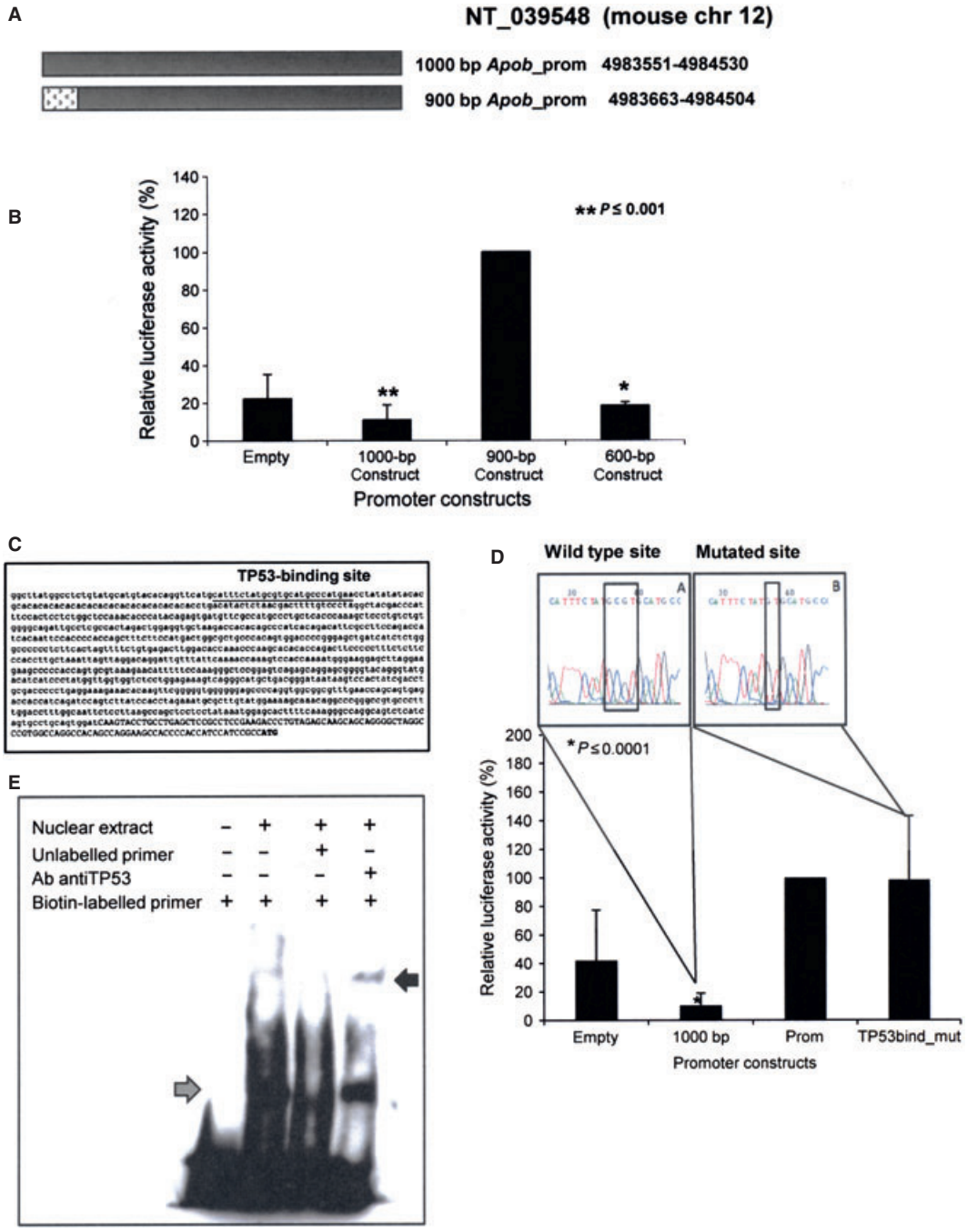

Figure 7 Identification of *cis* regulatory regions in mouse *Apob* promoter. (A) Map of the different constructs inserted in the pGL3 basic vector. (B) Results of the luciferase activity for the different constructs, compared to the predicted promoter (900-bp construct). Stars indicate the statistically significant different activities compared to the 900-bp construct. (C) Identification of p53 binding site in mouse *ApoB* promoter. (D) Luciferase assay of the wild-type and mutant promoter regions (see text for details). (E) EMSA assay showing the binding of p53 (fourth lane, black arrow); gray arrow, complex forming in presence of Neuro2a nuclear extracts.

Although the downstream factors involved in the final regulation of *Apob* expression are still largely not known, we have proven that p53 is a key regulatory molecule for *Apob* expression. Luciferase and EMSA assays have shown that p53 acts as a repressor directly binding to the mouse promoter region of *Apob*. Because MAPK P38 kinase induces p53 phosphorylation,[30] it is likely that the GDNF/Ret pathway acti-

vates the MAPK P38, which releases p53 from the promoter region thereby inducing *Apob* transcription. In support of this possibility recent data have indicated that p53 counteracts Ret activity during kidney development.[31]

Furthermore, we investigated whether or not *Apob* activation was related to enteric neuronal development by assessing HuD expression in GDNF-treated Neuro2a. Hu proteins are RNA-binding proteins (also referred to as HuD, HuC, HuR, and Hel-N1) that share sequence homology with the embryonic-lethal abnormal vision RNA-binding protein of *Drosophila*. In particular, HuD is exclusively expressed in neurons, where it exerts a crucial role in neuronal development and survival.[32,33] In the present experiments, we provide evidence that HuD protein levels are linked to *Apob* expression. GDNF treatment of Neuro2a cells transfected with a control vector evoked *Apob* expression which was associated with HuD increase; in contrast, in *Apob* silenced Neuro2a cells GDNF did not increase HuD protein. Our data in Neuro2a cells provide support to the concept that *Apob* can contribute to enteric neuron differentiation and survival via HuD expression. In this line, early data by our group showed that anti-HuD antibodies incubated with primary culture of mature myenteric neurons evoked an apoptotic effect.[34]

In addition to the *in vitro* results, homozygous mice knock-in for the HSCR-associated Ret^{C620R} had a significant decrease of *Apob* expression compared to wild-type animals. In contrast, heterozygous mice, which have a normal differentiation and organization of the ENS, displayed a marked increase in *Apob* expression. The latter effect may be promoted by the wild-type Ret, to compensate for the loss of function of the mutated Ret isoform. In this line, recent data from our group showed that *APOB* mRNA and protein expression are increased in cells of the immune system and in serum of patients with chronic intestinal pseudo-obstruction compared to healthy controls (Bonora E and De Giorgio R, unpublished observation). The evidence that APOB expression can be detected in these cells is in agreement with data showing RET expression in the lymphoid tissues of the gut, where this receptor exerts an important regulatory function for Peyer's patch formation.[35] Accordingly, in this study, APOB immunostaining was selectively detected in cells, most likely of the immune system, distributed throughout the *lamina propria* of the normal adult colon.

To our knowledge, this is the first report dealing with Ret-related *Apob* expression in both *in vitro* (Neuro2a cells) and *in vivo* (Ret C620R knock-in mouse) models. More broadly, it may be proposed that RET-dependent *APOB* expression exerts a major role in regulating different molecular pathways required for enteric neurogenesis and other tissue development. Likewise RET, also APOB may influence the immunoregulatory function in the digestive system, although this exciting aspect clearly deserves further investigation. The data herein presented provide a conceptual framework to evaluate APOB in patients with severe functional gastrointestinal disorders characterized by an impaired motility and transit.

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DISCLOSURE

The authors declare no competing interests.

AUTHOR CONTRIBUTION

CE performed the research, analyzed and interpreted the data and wrote the paper; FB, LMP, MR performed the research; AP, IS, AG, MV analyzed and interpreted the data; MS, GR, EB designed the experimental plan and wrote the paper; RDeG and VS revised the whole manuscript and contributed to experimental analysis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cell morphology classification. Cells were classified according to the presence and shape of neurites as follows: undifferentiated, round-shaped cells (A); with one neurite (monopolar) (B), with two opposite neurites (bipolar) (C); with two opposite and branched neurites (branched-bipolar) (D), with many neurites (star cells) (E); with one prevalent branched neurite (branched) (F).

Figure S2. (A) Western blot analysis of GDNF-treated Neuro2a in absence or presence (last 4 lanes) of Triciribine V, an Akt inhibitor. (B) qRT-PCR for *Apob* expression in GDNF-treated Neuro2a in presence of Triciribine V. (C) Western blot analysis of GDNF-treated Neuro2a in absence or presence (last 4 lanes) of PD98059, a specific ERK inhibitor. (F) qRT-PCR for *Apob* expression in GDNF-treated Neuro2a in presence of PD98059.

Figure S3. Luciferase activity assay for the different constructs, compared to the predicted promoter (900 bp construct), in absence of GDNF stimulation.

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