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ORIGINAL ARTICLE

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NDRG4, an early detection marker for colorectal cancer, is specifically expressed in enteric neurons

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Abstract

Background: Promoter methylation of *N-myc Downstream-Regulated Gene 4* (NDRG4) in fecal DNA is an established early detection marker for colorectal cancer (CRC). Despite its connection to CRC, NDRG4 is predominantly studied in brain and heart, with little to no knowledge about its expression or role in other organs. In this study, we aimed to determine the whole-body expression of NDRG4, with a focus on the intestinal tract.

Methods: We investigated NDRG4 expression throughout the body by immunohistochemistry, Western Blotting and *in situ mRNA* hybridization using tissues from *NDRG4* wild-type, heterozygous and knockout mice and humans. In addition, we explored cell-specific expression of NDRG4 in murine whole-mount gut preparations using immunofluorescence and confocal microscopy.

Key Results: NDRG4 is specifically expressed within nervous system structures throughout the body. In the intestinal tract of both mouse and man, NDRG4 immunoreactivity was restricted to the enteric nervous system (ENS), where it labeled cell bodies of the myenteric and submucosal plexuses and interconnecting nerve fibers. More precisely, NDRG4 expression was limited to neurons, as NDRG4 always co-localized with HuC/D (pan-neuronal marker) but never with GFAP (an enteric glial cell

Abbreviations: BDM1, brain development-related molecule 1; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CRC, colorectal cancer; ENS, enteric nervous system; FDA, U.S. food and drug administration; GFAP, glial fibrillary acidic protein; GI, gastrointestinal; NDRG4, N-myc downstream-regulated gene; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; Ret, receptor tyrosine kinase; SMAP-8, smooth muscle-associated protein 8; SNAP, soluble NSF attachment protein; VAMP, vesicle-associated membrane protein.

marker). Furthermore, NDRG4 was expressed in various neuropeptide Y positive neurons, but was only found in a minority (~10%) of neurons expressing neuronal nitric oxide synthase.

Conclusions and Inferences: NDRG4 is exclusively expressed by central, peripheral and enteric neurons/nerves, suggesting a neuronal-specific role of this protein. Our findings raise the question whether NDRG4, via the ENS, an understudied component of the tumor microenvironment, supports CRC development and/or progression.

KEYWORDS

biomarker, colorectal cancer, enteric nervous system, myenteric plexus, NDRG4

1 | INTRODUCTION

Previously, we identified *N-myc Downstream-Regulated Gene 4 (NDRG4)* promoter CpG island methylation as a promising early detection marker for colorectal cancer (CRC),[1] an observation that has been independently validated.[2,3] *NDRG4* is one of the molecular markers of the FDA-approved, multi-target stool DNA test (Cologuard®, Exact Sciences Corporation, Madison, USA), which detects significantly more cancers than the leading fecal immunochemical test (FIT), and is currently used as a screening modality in the USA.[3] Surprisingly, given its connection to CRC, the expression pattern and function(s) of NDRG4 in the gut and its role in CRC carcinogenesis remain to be elucidated.

NDRG4, also known as *SMAP-8* and *BDM1*, is one of the four members (*NDRG1-4)* of the *NDRG* gene family, a group of genes involved in cell proliferation, differentiation, development and stress.[4] The encoded proteins of this family, characterized by an α/β hydrolase fold, share 52%-65% sequence homology and are highly conserved in evolution in various species.[5,6] The *NDRG4* gene was originally characterized in humans and encodes three isoforms: NDRG4H (41 kDa), NDRG4B (37 kDa) and NDRG4B^{var}(39 kDa).[5] Although all three human isoforms have been identified in mouse and rat brain, the latter reveals an additional fourth isoform (45 kDa) encoded by the rat ortholog of human *NDRG4: 'SMAP-8/BDM1'*, while the mouse orthologs correspond to NDRG4B and NDRG4B^{var}.[7,8] In contrast to the well-described ubiquitous expression of NDRG1, NDRG2 and NDRG3, abundant expression of NDRG4 is predominantly observed in brain and heart, suggesting an important role of NDRG4 in these organs.[5–10] In fact, in the central nervous system (CNS), *NDRG4* is required for normal morphogenesis of the mouse and zebrafish brain and to protect against neurological deficits by maintaining physiological levels of brain-derived neurotrophic factor (BDNF).[7,10] *NDRG4* is essential for proper neurite outgrowth, neural functions *in vitro* and myelination of axons in zebrafish.[11,12] Consistent with these observations, the Alzheimer diseased brain, which is characterized by neuronal degeneration, shows reduced levels of *NDRG4*.[5] Furthermore, proper morphogenesis of the mouse and zebrafish heart is regulated by *NDRG4* and *NDRG4* deficiency is associated with weak contractility and reduced heart rate in zebrafish.[10,13] Similarly, variations in locus 16q21 near *NDRG4* have been associated with prolongation of the QT interval, a risk factor for sudden cardiac death.[14]

KEY POINTS

- Despite its excellent performance as biomarker, the expression and function of NDRG4 in the gut and its role in CRC carcinogenesis are unknown
- NDRG4 is specifically expressed in neurons of the enteric nervous system
- The specific expression of NDRG4 unravels a novel neuron-specific role of this protein and opens the discussion of whether the ENS contributes to CRC carcinogenesis

Nevertheless, the molecular mechanisms by which NDRG4 affects the above-described phenomena are still unknown.

As the expression and functional role(s) of NDRG4 outside the heart and brain are largely unstudied, we examined the overall whole-body expression of NDRG4, while focusing on the intestinal tract as a first step in understanding the role(s) of NDRG4 in the gut. Therefore, we determined NDRG4 expression according the two main specificity criteria as described by Pradidarcheep et al.,[15] by means of immunohistochemistry, *in situ mRNA* hybridization and Western blotting using human, *NDRG4* wild-type and *NDRG4* knockout mouse tissues. In addition, we investigated cell-specific expression of NDRG4 in more depth in murine whole-mount gut preparations using immunofluorescence.

2 | MATERIALS AND METHODS

2.1 | Human

Formalin-fixed, paraffin-embedded human normal colon tissues of CRC patients (n=5) were retrospectively collected from the archive of the Department of Pathology of the Maastricht University Medical Center. Written informed consent was obtained from all study participants and the process adhered to local ethics guidelines.

2.2 | Mice

NDRG4 wild-type (*NDRG4*$^{+/+}$), heterozygous (*NDRG4*$^{+/-}$) and knock-out (*NDRG4*$^{-/-}$) mice (C57BL/6 genetic background) were kindly provided

by Prof. Baldwin (Vanderbilt University Medical Center)[16] and characterized by genotyping PCR (See "Genotyping"). Animals were age- and gender matched and housed in groups of 3-5 under standard conditions having free access to food and water. One-year old *NDRG4*$^{+/+}$, *NDRG4*$^{+/-}$ and *NDRG4*$^{-/-}$ mice were sacrificed, tissues were harvested and either snap-frozen for protein isolation or fixed in 4% formaldehyde for immunohistochemistry. In addition, 2 month old *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ mice were sacrificed, followed by removal of brain and intestines for immunostainings on brain and whole-mount gut preparations.[17] Animal experiments were approved by the Committee of Animal Welfare of Maastricht University and performed according to Dutch regulations.

2.3 | Genotyping

To identify carriers of the *NDRG4* wild-type and deletion allele, purified DNA was examined by PCR. Genomic DNA was purified with the Gentra Puregene Mouse tail kit (Qiagen, Maryland, USA) according to manufacturer's instructions. For the detection of each allele, a PCR was performed with the *NDRG4* primer mix listed in Table 1. The PCR reaction mix contained 100 ng genomic DNA, 10 μL REDExtract-N-Amp PCR Reaction Mix (REDExtract-N-Amp™ Tissue PCR Kit, Sigma-Aldrich, St. Louis, MO, USA) and 0.8 μL primer mix (10 μM) in a final volume of 20 μL. The PCR was performed using the Biorad T100™ thermal cycler (Biorad) with the following conditions: (i) initial incubation: 94°C for 3 minutes, (ii) 35 cycles: 94°C for 30 seconds—60°C (annealing temperature) for 30 seconds—72°C for 1 minutes, and (iii) final elongation: 72°C for 10 minutes. PCR products were detected in a 1.5% (w/v) agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer. The *NDRG4* wild-type locus is identified by a 203 and 957 bp fragment, whereas a 320 bp band identifies the deletion allele.

2.4 | Protein isolation and western blotting

Brain, heart and colon tissues from *NDRG4*$^{+/+}$, *NDRG4*$^{+/-}$ and *NDRG4*$^{-/-}$ mice were homogenized in RIPA buffer (Pierce Technology, Rockford, Illinois, USA) containing protease inhibitors (1 "complete" pill/50 mL, Roche, Mannheim, Germany), and resolved in SDS-gel electrophoresis. Protein transfers were probed overnight (4°C) with mouse anti-human NDRG4, clone 2G3 (1:500; H00065009-M01; Abnova, Taipei City, Taiwan), rabbit anti-human NDRG4 (1:1000; #9039; Cell Signaling, Leiden, The Netherlands) and β-actin (1:200 000; Sigma-Aldrich). Bound antibodies were visualized by an HRP-linked secondary anti-rabbit or anti-mouse antibody (1 hour, RT, Cell Signaling) and chemiluminescence (ECL, Pierce Technology, Rockford, Illinois, USA).

2.5 | Immunohistochemistry

Three μm thick paraffin sections of every organ of *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ mice and human colon were deparaffinized in xylene and rehydrated in graded alcohols. To quench endogenous peroxidase activity, the slides were incubated with 0.3% hydrogen peroxide in methanol for 20 minutes. Antigen retrieval was performed by boiling the sections in Tris-EDTA buffer (pH 8.0; Klinipath, Duiven, The Netherlands) or Dako target retrieval solution (pH 6.0; Dako, Santa Clara, CA, USA), followed by blocking nonspecific antibody binding with PBS containing 20% fetal bovine serum and 0.1% Tween. Sections where incubated overnight at 4°C with the primary antibodies: mouse anti-human NDRG4, clone 2G3 (1:500, Abnova) or rabbit anti-human NDRG4 antibody (Mouse brain and other organs 1:500 and 1:250, resp.; Human colon, 1:25; Cell Signaling) diluted in PBS/0.5%BSA/0.1%Tween. After incubation with the biotinylated anti-mouse secondary antibody (1:250; RPN1001v1; Amerscham Biosciences, Piscataway, NJ, USA) followed by the Avidin-Biotin Complex detection method (ABC; 1:500; Dako Cytomation, Glostrup, Denmark) or incubation with horseradish peroxidase-conjugated anti-rabbit IgGs (poly-HRP, Immunologic, Duiven, The Netherlands), bound antibodies were visualized using 3,3'-diaminobenzidine (DAB, Dako) as a chromogen (brown precipitate). Slides were counterstained with hematoxylin, dehydrated and mounted. In addition, to diminish recognition of endogenous mouse immunoglobins by the mouse primary antibody, slides were subsequently stained with the mouse anti-human NDRG4 antibody, clone 2G3 (1:500; H00065009-M01; Abnova) using the Vector® M.O.M.™ Immunodetection Kit according to manufacturer's instructions (Vector Labs, Burlingame, CA, USA). Negative controls for primary antibodies were monitored by staining of *NDRG4*$^{-/-}$ tissues and omission of primary antibodies from *NDRG4*$^{+/+}$ slides. Images were acquired at RT using a Nikon DMX1200 digital camera and the ACT-1 v2.62 software from Nikon Corporation (Amsterdam, The Netherlands).

2.6 | Immunofluorescence

Immunostainings were performed on brain slices and whole-mount gut preparations as previously described.[17] Briefly, ileum and colon of *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ mice were collected, opened along the mesenteric border, stretched and pinned flat with insect pins (0.2 mm, Agar Scientific, Stansted, UK) in a Sylgard-lined dissection dish (Sylgard 184 Elastomer, Down Corning, Auburn, MI, USA) with Krebs solution bubbled with 95% O_2-5% CO_2. The mucosal and submucosal layers were removed, tissues fixed for 30 minutes (4°C) in paraformaldehyde (4% in PBS) and rinsed in PBS. To visualize the myenteric and

TABLE 1 PCR primers for genotyping

Target	Forward primer	Reverse primer	Annealing temp. (°C)
NDRG4 mouse			
NDRG4 4HLOX1	TAGGCAGGGGCAGGTGGGTTTGT		60
NDRG4 4HLOX2		GGCGTCCTGATGTCATGTTCCTGT	60
NDRG4 4H776		GCTCCCACTCCAATGCCAATC	60

submucosal plexus the circular or longitudinal muscle layer, respectively, were peeled. Brain and gut tissues were permeabilized in 0.5% tritonX-100 and incubated in blocking solution (4% goat/donkey serum), followed by an overnight incubation (4°C) with the primary antibodies diluted in blocking solution: rabbit anti-NDRG4 (1:500, Cell Signaling), chicken anti-GFAP (1:5000; Abcam, Cambridge, UK), human anti-HuC/HuD (1:500; Invitrogen Life Technologies, Rockford, Illinois, USA), sheep anti-neuronal NO-synthase (1:400; Santa Cruz Biotechnologies, Delaware, CA, USA), rabbit anti-calretinin (1:2000; Chemicon, Darmstadt, Germany), rat anti-neuropeptide Y (1:500; Eugene Tech International Allendale, NJ, USA) for whole-mount gut preparations; rabbit anti-NDRG4 (1:500; Cell Signaling) and mouse anti-human NeuN (1:100, Chemicon) for brain slides. After rinsing, tissues were incubated for 2 hours with the fluorescently labeled secondary antibodies: anti-human Alexa594, anti-mouse Alexa594, anti-rabbit Alexa488, anti-sheep Alexa488, anti-rat Alexa488 (all 1:1000; Molecular probes, Invitrogen, Belgium), anti-rabbit AMCA or anti-chicken AMCA (both 1:250; Jackson Immuno Research Labs, West Grove, PA, USA). Samples were mounted with Vectashield™ mounting medium (Vector Labs, Burlingame, CA, USA). Preparations were imaged with a Zeiss LSM780 confocal microscope (Cell imaging Core, KU Leuven).

2.7 | *In situ mRNA* hybridization

In situ mRNA hybridization was performed on paraffin-embedded brains and intestinal Swiss rolls of *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ mice using digoxigenin (DIG) labeled riboprobes (DIG RNA Labeling Kit, SP6/T7, Roche) for NDRG4 nucleotide positions 1269-1777 (NDRG4-A) and 1811-2343 (NDRG4-B) of mouse NDRG4 (NM_145602, Table 2).[7] Four µm sections were dried upright, deparaffinized, rehydrated and washed. Tissues were permeabilized with 0.1% pepsin in 0.2N HCl for 5 minutes (37°C) and post-fixed in 4% paraformaldehyde (4°C). Fixed sections were then treated twice with 100 mM glycine in 1xPBS and prehybridized (37°C) for 45 minutes in prehybridization buffer (2× SCC, 50% (v/v) deionized formamide, 40% (v/v) DEPC-treated H_2O). *NDRG4* mRNA was detected overnight at 37°C with the preheated hybridization buffer (2× SCC, 50% (v/v) formamide, 10% dextran sulphate, 10 mM DTT,7,5% DEPC-H_2O, 1× Denhardts solution, 1 mg/mL yeast tRNA, 1 mg/mL denatured and sheared salmon sperm DNA) containing a mixture of both antisense or sense probes (1 µg/mL). After a serial wash in descending concentrations (2×, 1× and 0.1×) of SCC buffer, tissue RNA was digested by RNase A (Roche), followed by a serial wash in ascending SCC concentrations (1× and 2×) and buffer 1 (100 mM Tris-HCl, 150 mM NaCl). Sections were then blocked for two hours (0.1% Triton X-100, 2% normal sheep serum in buffer 1) and incubated with 1:100 anti-DIG alkaline phosphatase Fab fragments (Roche) for 1 hour at 37°C. Following washes in buffer 1 and 2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$), the chromogenic substrates nitro-blue tetrazolium chloride and 5-bromo-4chloro-3indolyl phosphate (Roche), dissolved in buffer 2 containing 5 mM levamisole, were used to detect hybridized alkaline phosphatase activity. Once the signal reached optimal intensity, the color reaction was stopped (10 mM Tris-HCl, 1 mM EDTA), sections were counterstained with nuclear fast red, clarified in tap water and mounted in imsol (1:3). Tissue sections of *NDRG4*$^{-/-}$ and *NDRG4*$^{+/+}$ mice immersed in antisense or sense riboprobes, respectively, served as negative controls. Images were acquired as described for IHC images.

3 | RESULTS

3.1 | The Cell Signaling antibody is able to specifically target NDRG4

We first characterized the specificity of both anti-NDRG4 antibodies using tissues of *NDRG4*$^{+/+}$, *NDRG4*$^{+/-}$ and *NDRG4*$^{-/-}$ mice in Western Blotting and immunohistochemistry (Figure 1). Western Blotting revealed that both antibodies could discriminate all known NDRG4 isoforms in various tissues, including proximal and distal colon of *NDRG4*$^{+/+}$ and *NDRG4*$^{+/-}$ mice (Figure 1A,B). However, successful deletion of *NDRG4* in *NDRG4*$^{-/-}$ mice was only confirmed after application of the Cell Signaling antibody. The specificity of the Abnova antibody, however, could not be confirmed, as the signal was still detectable in *NDRG4*$^{-/-}$ tissues and several non-specific bands of about 50-55 kDa were recognized. Non-specific recognition of NDRG4 by the Abnova antibody was also confirmed by the similar staining pattern observed in all examined tissues of *NDRG4*$^{-/-}$ and *NDRG4*$^{+/+}$ mice (Figure 1C). The Cell Signaling antibody on the other hand, detected NDRG4 in *NDRG4*$^{+/+}$, but not in *NDRG4*$^{-/-}$ tissues (Figure 1D). Thus, only the Cell Signaling antibody (#9039) is able to specifically target NDRG4 and was therefore further used to comprehensively study the expression of NDRG4 throughout the body and specifically within the gut.

3.2 | NDRG4 is specifically expressed within the enteric nervous system of both mouse and man

Characterization of NDRG4 expression in the gastrointestinal (GI) tract (ie, stomach, small and large intestine) of *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ mice using immunohistochemistry, revealed that NDRG4 is specifically expressed within the nervous system of the gut, ie, the enteric nervous system (ENS; Figure 2).[18] More precisely, NDRG4 is expressed in the cytoplasm of cell bodies inside ganglia of the myenteric plexus,

TABLE 2 Probes for *in situ mRNA* hybridization

Target	Forward primer	Reverse primer	Annealing temp. (°C)
NDRG4 mouse			
NDRG4-A	TTATTTAAAAAGAAATGAGGGGATC	TTGCCTCAGGGTGGGACAA	58.5 (F)-60.2 (R)
NDRG4-B	GTTAAAATGTTGATTGCTGTGTATGC	ACTCCAGAGCAGTCTAGAAATGGC	60.5 (F)-60.9 (R)

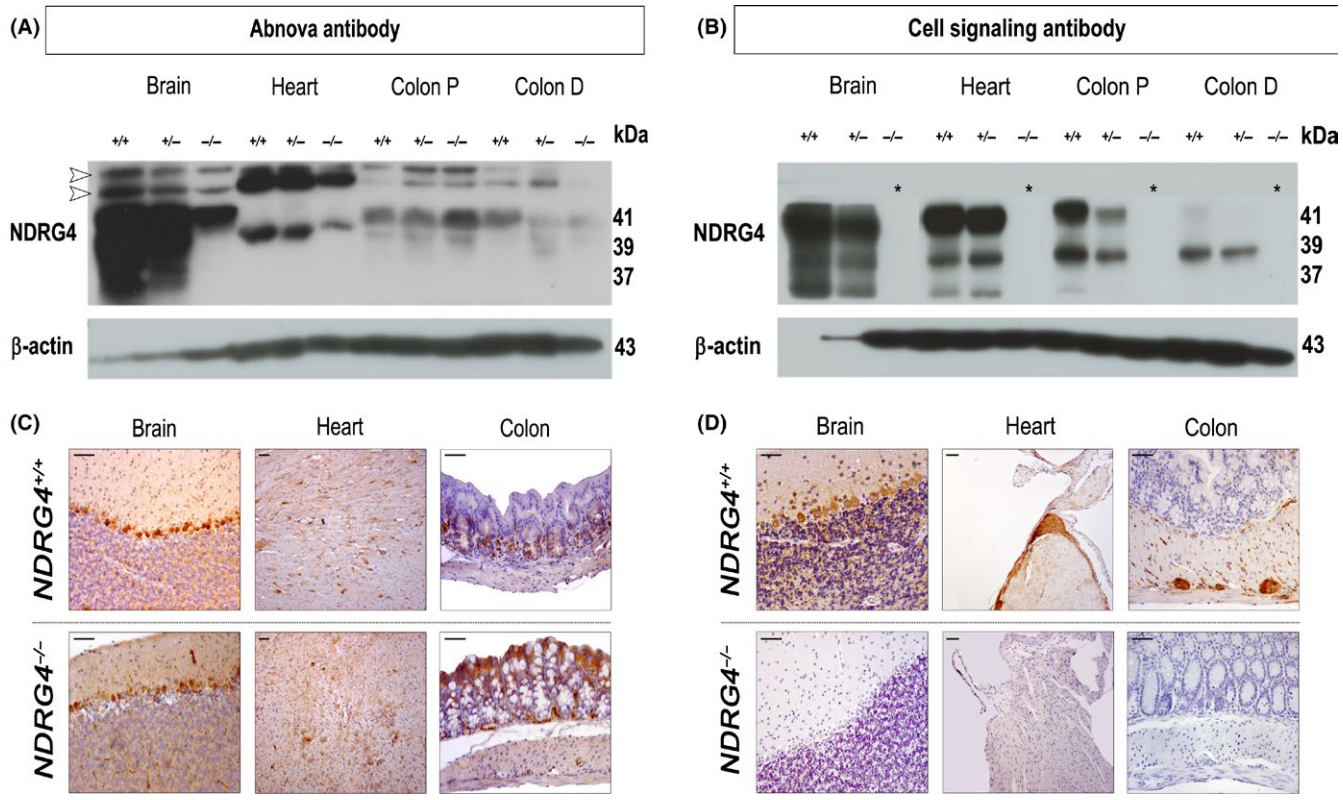

FIGURE 1 *The Cell Signaling, but not the Abnova antibody specifically targets NDRG4.* (A,B) Western Blotting analysis of *NDRG4*$^{+/+}$, *NDRG4*$^{+/-}$ and *NDRG4*$^{-/-}$ brain, heart and colon (P, proximal; D, distal) using the Abnova (A) and Cell Signaling antibody (B). Both antibodies recognize all three well-known isoforms: NDRG4B (37 kDa), NDRG4B^{var} (39 kDa) and NDRG4H (41 kDa) in *NDRG4*$^{+/+}$ and *NDRG4*$^{+/-}$ tissues. Open triangles indicate non-specific bands (50-55 kDa) identified by the Abnova antibody (A). * indicates successful detection of NDRG4 ablation only after application of the Cell Signaling antibody (B). β-actin is used as loading control. (C,D) Brain, heart and colon sections from 1-year old *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ mice were subjected to IHC with the Abnova (C) and Cell Signaling antibody (D). Positive signals (brown) detected by the Abnova antibody are not affected by absence of *NDRG4* and differ from the highly selective staining pattern produced by the Cell Signaling antibody. Scale bars, 50 μm in C and D

located between the outer longitudinal and inner circular muscle layer along the entire GI tract and within the ganglia of the submucosal plexus (Figure 2A). The nerve fiber bundles connecting the ganglia in both plexuses also showed NDRG4 expression. Furthermore, the nerves innervating the outer and inner muscularis externa, muscularis mucosae and those projecting into the mucosa also showed NDRG4 immunoreactivity. As explained above, specificity of the antibody was monitored by NDRG4 immunohistochemistry on the gut of *NDRG4*$^{-/-}$ mice. Representative immunohistochemical images (Figure 2A) show that the intestinal tract of *NDRG4*$^{-/-}$ mice still contains all the ENS structures described above, ie, ganglia of the myenteric and submucosal plexus and interconnecting nerve fibers, but does not display NDRG4 immunoreactivity, confirming the absence of NDRG4 expression. Furthermore, *in situ mRNA* hybridization confirmed that NDRG4 is specifically expressed within the ENS of *NDRG4*$^{+/+}$ mice, but not in *NDRG4*$^{-/-}$ mice (Figure 2A). Similar to that described in mouse, human intestinal specimens displayed NDRG4 immunoreactivity in the ganglia of both plexuses, in nerve fibers connecting these ganglia and within the fibers innervating both muscle layers of the muscularis externa and muscularis mucosae, as shown in Figure 2B. Compared to mouse, the mucosal projecting nerve fibers showed more robust NDRG4 immunoreactivity. Together these data indicate that NDRG4 is specifically expressed within the ENS of mice and humans.

3.3 | NDRG4 is expressed within enteric neuronal cells

Whole-mount preparations of the submucosal and myenteric plexuses of the *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ small and large intestine were used to explore what types of enteric neurons express NDRG4. Since similar results were observed in above-mentioned whole-mount preparations, only data from the colonic myenteric plexus are shown (Figure 3). Immunofluorescent triple labeling revealed that within the gut of *NDRG4*$^{+/+}$ mice, NDRG4 is exclusively expressed within the cytoplasm of enteric neurons, as NDRG4 positive cells were always labeled for the pan-neuronal marker HuC/D, but never co-expressed the glial cell marker GFAP (Figure 3A). Even though NDRG4 localized in enteric neurons throughout all areas of the ENS, not all enteric neurons (HuC/D positive) expressed NDRG4 (Figure 3A). To further investigate visual differences in the organization of the plexuses between *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ mice, the two main neuronal subsets, positive for the neuronal markers nitric oxide synthase (nNOS; inhibitory

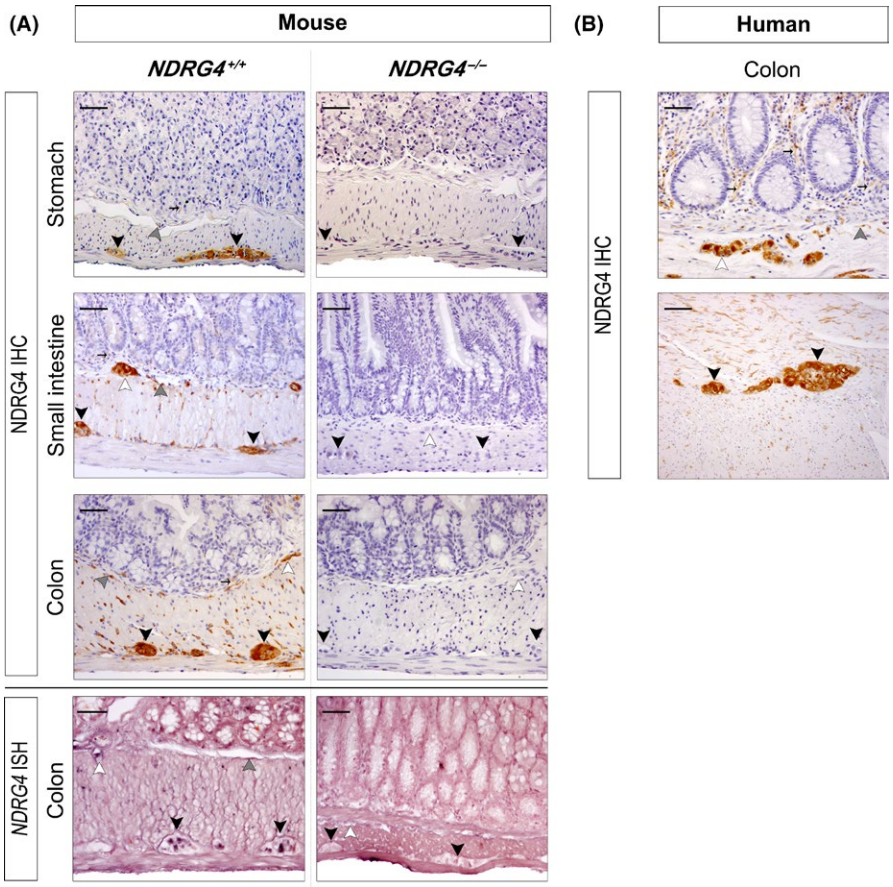

FIGURE 2 *NDRG4 is localized within the ENS of mouse and man.* A, Representative immunohistochemical staining (IHC) of the GI tract (ie, stomach, small intestine (duodenum) and colon) and colonic *in situ mRNA* hybridization (ISH) of *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ *mice*. NDRG4 RNA and protein is expressed in myenteric and submucosal ganglia (black and open arrowhead, respectively.), within interconnecting nerve fibers throughout the plexus, muscularis externa, muscularis mucosae (gray arrowhead) and mucosae (black arrow). Deletion of *NDRG4* is confirmed at protein and RNA level. B, NDRG4 expression in human colonic specimens is analogous to mouse, with more robustly stained mucosal projecting nerve fibers (black arrows). Scale bars, 50 μm in A; and 100 μm in B

motor neurons and descending interneurons) or calretinin (excitatory motor neurons), were studied in combination with HuC/D (Figure 3B). The overall neuronal population in *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ colon is fairly similar as indicated by analogous HuC/D positivity and calretinin and nNOS staining (Figure 3B). Nevertheless, our preliminary observations reveal that the NDRG4 and nNOS positive population were almost completely different from each other (Figure 3C). More precisely, neurons that express NDRG4 are almost exclusively nNOS negative: only 3% of the NDRG4 positive enteric neurons also expressed nNOS and vice versa, only 10% of the nNOS population was immunoreactive for NDRG4. Finally, to investigate if NDRG4 is expressed by secretomotor neurons, the co-localization of NDRG4 with the secretomotor neuron marker NPY was studied. Overlap of NDRG4 with NPY was observed in neuronal cell bodies of the intestinal plexuses of *NDRG4*$^{+/+}$ mice (Figure 3D). In conclusion, NDRG4 is expressed in different subsets of enteric neurons.

3.4 | NDRG4 is expressed by neuronal cells in the central and peripheral nervous system

Finally, we confirmed that NDRG4 is specifically expressed within the central nervous system (Figure 4A). Neurons in the gray matter structures of the cerebrum (eg, hippocampus and thalamus), cerebellum (eg, Purkinje and molecular layer) and spinal cord of *NDRG4*$^{+/+}$ mice showed specific, predominant cytoplasmic expression of NDRG4, while the white matter, ependymal cells and meninges were negative for NDRG4. In the peripheral nervous system, we observed a similar staining pattern (Figure 4B). More specifically, the cytoplasm of ganglia situated in close proximity of every organ, ie, prevertebral ganglia, and of the ventral and dorsal root ganglia showed strong NDRG4 reactivity. Furthermore, the tiny nerve fibers throughout peripheral organs itself (eg, in the bronchial branch) also showed strong NDRG4 immunoreactivity. Hence, NDRG4 is almost exclusively expressed in the central, peripheral and enteric nervous system.

4 | DISCUSSION

Our previous work has shown that the biomarker potential of *NDRG4* is associated with a putative tumor suppressor role *in vitro*.[1] In addition, we and various independent groups, described that NDRG4 expression in the intestinal epithelium decreases in the transition towards CRC.[1,19,20] However, all these studies used the commercially available monoclonal mouse anti-human NDRG4 antibody from Abnova (clone 2G3, H00065009-M01), which has, to our knowledge, never been validated to specifically target NDRG4. In this study, we investigated the whole-body expression of NDRG4, focusing on the GI tract, using two commercially available antibodies: the monoclonal mouse anti-human NDRG4 from Abnova (clone 2G3, H00065009-M01)[1,17,20,21] and polyclonal rabbit anti-human NDRG4 from Cell Signaling (#9039)[22,23] according to the specificity criteria,[15] as a critical first step in understanding the role(s) of NDRG4.

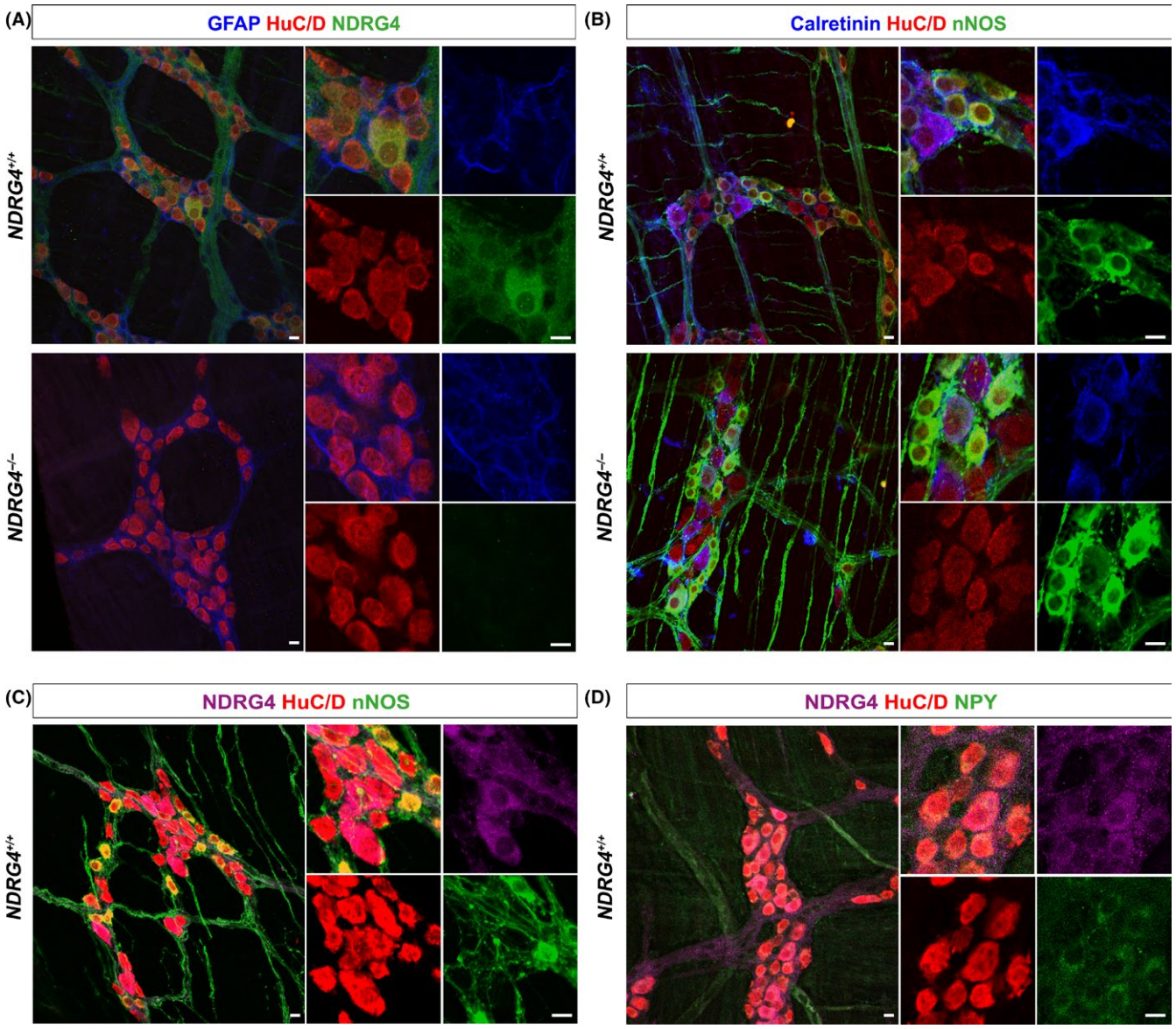

FIGURE 3 *NDRG4 is expressed in different subsets of enteric neurons*. (A-D) Representative fluorescently labeled whole-mount preparations of colonic myenteric plexus of *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ mice. Scale bars, 5 µm. A, NDRG4 always co-localized with HuC/D (green vs red) but never with GFAP (green vs blue). *NDRG4*$^{-/-}$ mice don't express NDRG4, but still possess enteric neurons (red) and glial cells (blue). B, Plexus of *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ revealed similarities in HuC/D, calretinin and nNOS positivity (red, blue and green, respectively). C, NDRG4 positive cells (purple) are almost never reactive to nNOS (green) and nNOS positive cells almost never express NDRG4. D, NDRG4 (purple) co-localized with NPY (green) in various cell bodies of secretomotor neurons

Western blotting and immunohistochemical analysis using tissues of *NDRG4*$^{+/+}$ and *NDRG4*$^{-/-}$ mice revealed that both the Abnova and Cell Signaling antibody could discriminate all three known human NDRG4 isoforms. However, the specificity for NDRG4 in *NDRG4*$^{-/-}$ mice was only confirmed using the Cell Signaling antibody. Given the high sequence similarity (52%-65%) between NDRG4 and its protein family members, it is likely that the Abnova antibody recognizes one of the other NDRG proteins. According to the phylogenetic tree, NDRG4 shares the closest relation with NDRG2, but either NDRG1, 2 or 3 might be detected, as their main isoforms are 43, 39, or 41 and 40 or 41, 5 kDa in size respectively.[1,4–7,10] Hence, this finding casts doubt on previously published statements that are based on the expression of NDRG4.

Using the validated Cell Signaling antibody, we explored the expression of NDRG4 throughout the body. We confirmed that NDRG4 is specifically expressed within neurons of the central nervous system (ie, brain and spinal cord)[7,9] and observed this neuronal-specific expression pattern through the whole-body. More depth investigation of the expression pattern of NDRG4 within the gut revealed the presence of the NDRG4B^{var} and NDRG4H isoform in murine colon and the specific expression of NDRG4 within the ENS. The ENS, the so-called brain of the gut, is an interconnected network of enteric neurons and glial cells, predominantly clustered in ganglia of the submucosal and myenteric plexuses, along the entire GI tract.[24] Interestingly, NDRG4 has already been identified with DNA microarray analysis

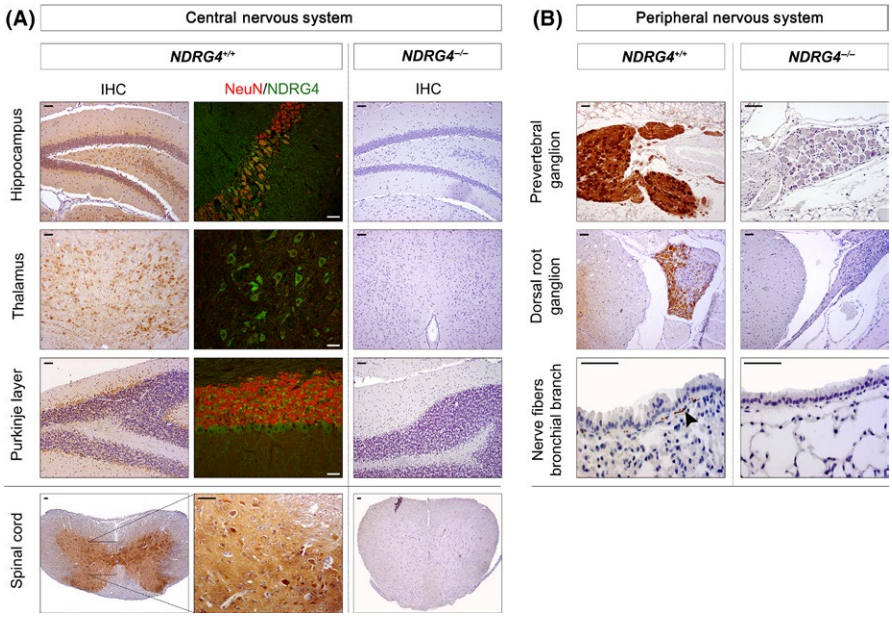

FIGURE 4 *NDRG4* is specifically expressed in the central en peripheral nervous system. (A) Immunohistochemical and immunofluorescent analysis of several areas of the one-year old mouse brain. Gray matter structures in the cerebrum and cerebellum of *NDRG4*$^{+/+}$ mice are positive for NDRG4 (green) and various regions show co-localization of NDRG4 with the neuronal marker NeuN (red): e.g. NeuN positive cells in the hippocampal cortex (pyramidal neurons and granule cells) and in the thalamus co-express NDRG4. Cerebral Purkinje cells, which are negative for NeuN, are strongly positive for NDRG4. The gray matter of the spinal cord also showed NDRG4 immunoreactivity within neuronal cell bodies (brown). NDRG4 is absent in all central nervous system structures in *NDRG4*$^{-/-}$ mice. (B) Representative images of peripheral tissues displayed NDRG4 expression in neurons, in prevertebral and dorsal root ganglia, and in nerves to, from and within every organ of the body (e.g. nerve fibers bronchial branch). White scale bars, 20µm; and black scale bars, 50µm in A and B.

and *in situ* hybridization as one of the significantly down-regulated genes in the aganglionic bowel of mice (*Ret*$^{k-/k-}$) and Hirschsprung's patients.[25,26] Though, here we describe for the first time that NDRG4 expression in the gut is restricted to enteric neurons as NDRG4 always co-localized with the pan-neuronal marker HuC/D, but never with the enteric glial cell marker GFAP. Together, our findings raise the question whether there is a role for the ENS in the development and/or progression of CRC.

While the reciprocal cross-talk between nerves and tumor cells, together with the concept of neoneurogenesis/axonogenesis[27] is nowadays a rapidly evolving field, very little research has been conducted to unravel the role of the ENS in CRC development/progression. Nevertheless, the importance of the ENS is emphasized by the broad range of (life-threatening) enteric neuropathies like Hirschsprung's disease that arise when alterations in the ENS occur. Furthermore, it has been described that impairments in the ENS can contribute to the development and severity of intestinal inflammation[28] and that inflammatory conditions of the gut are often associated with changes in the ENS.[29,30] A growing body of evidence demonstrates that proper functioning of the ENS, ensured by the interplay between enteric neurons and enteric glial cells, is pivotal to maintain the integrity of the intestinal epithelial barrier to regulate gut homeostasis. Neurons, like enteroendocrine cells, synthesize various neurotrophic factors and messenger molecules, which are packaged in vesicles and released in exocytotic processes. The process of exocytosis allows a cell to communicate with neighboring cells, ie, other neurons, glia, muscle, endothelial, immune and epithelial cells.[22] It is well-established that several (enteric) neurotransmitters, eg, acetylcholine, vasoactive intestinal peptide and substance P, can target intestinal stem cells.[31] These data suggest a potential role for the ENS in the development/progression of CRC.

NDRG4 has been implicated in the route of vesicular transport. The interaction of NDRG4 with Blood vessel epicardial substance (Bves) has been shown to regulate docking of VAMP-3 (SNARE-protein) positive vesicles to the cell surface and the subsequent cargo delivery (eg, fibronectin).[32] Similarly, NDRG4 controls vesicle membrane fusion during exocytosis as *NDRG4* knockdown has been associated with a sharp reduction in the level of another vesicle-SNARE protein, SNAP25.[12] The SNARE proteins, including SNAP25, are complexes that are essential in promoting subcellular trafficking, vesicular fusion and subsequent exocytotic release of neurotransmitters.[33] Hence, it is reasonable that NDRG4, like NDRG1 and NDRG2, is able to modulate subcellular vesicle trafficking and exocytotic release of neuromediators,[34,35] thereby regulating neurotransmission and the subsequent targeting of the epithelial layer.

In summary, we previously observed that the recognition of *NDRG4* promoter methylation in fecal DNA is a valuable tool for the non-invasive detection of CRC and we identified NDRG4 as a tumor suppressor gene in CRC whose epithelial expression decreases from normal mucosa to CRC. However, using the validated Cell Signaling antibody, we found that NDRG4 is specifically expressed within central, peripheral and enteric neurons but not in epithelial cells. Although it still remains to be elucidated how the expression pattern of NDRG4 is linked with its biomarker performance for CRC, validating our

hypothesis that NDRG4 can influence CRC development and/or progression via the ENS will have a major impact on current concepts in CRC research.

DISCLOSURE

The authors disclose no conflicts.

AUTHOR CONTRIBUTION

NV contributed to the literature search, study concept and design, data collection, data analysis and data interpretation, generation of figures and writing of the manuscript; MHFML contributed to the literature search, histological data collection and interpretation, and writing of the manuscript; MJG contributed to histological data collection, analysis and interpretation, and critical revision of the manuscript; GR contributed towards critical revision of the manuscript; KLD contributed to data collection; WB contributed to data collection and generation of figures; KADW contributed to data collection and interpretation; AG contributed towards data collection and generation of figures; XQ contributed towards critical revision of the manuscript; HPJS contributed towards data collection and interpretation; BPFR contributed towards critical revision of the manuscript; SHB contributed to data analysis and interpretation and critical revision of the manuscript; KAS, RMWH and MvE contributed towards data analysis and interpretation and critical revision of the manuscript; PvB contributed to data collection, data analysis and interpretation, generation of figures and critical revision of the manuscript; VM obtained funding and contributed to study concept and design, data analysis and data interpretation, and writing of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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