

A combined literature and in silico analysis enlightens the role of the NDRG family in the gut.

Nathalie Vaes^a, Simone L. Schonkeren^a, Erwin Brosens^b, Alexander Koch^a, Conor J. McCann^c, Nikhil Thapar^c, Robert M.W. Hofstra^b, Manon van Engeland^a, Veerle Melotte^{a,b}

Affiliations of authors:

^aDepartment of Pathology, GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center, The Netherlands.

n.vaes@maastrichtuniversity.nl, s.schonkeren@student.maastrichtuniversity.nl, a.koch@maastrichtuniversity.nl,
m.vanengeland@maastrichtuniversity.nl, veerle.melotte@maastrichtuniversity.nl

^bDepartment of Clinical Genetics, University of Rotterdam, EMC, Rotterdam, The Netherlands.

e.brosens@erasmusmc.nl, r.hofstra@erasmusmc.nl

^cStem Cells and Regenerative Medicine, UCL Great Ormond Street Institute of Child Health, London WC1N, UK.

conor.mccann@ucl.ac.uk, n.thapar@ucl.ac.uk

Address for correspondence:

Dr. V. Melotte, Dept. of Pathology, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Center, P.O. Box 616, 6200 MD Maastricht, The Netherlands, T: +31-4338872210, F: +31-43-3876613

E-mail: veerle.melotte@maastrichtuniversity.nl

Highlights

- Diverse expression: epithelium, NDRG1/2; enteric neurons, NDRG4; unknown, NDRG3.
- Reduced NDRG2 & 4 levels in CRC are mainly caused by DNA promoter hypermethylation.
- NDRG1, 2 and 4 are tumor/metastasis suppressor genes in CRC.
- NDRG1, 2 and 4 are potential biomarkers for CRC.
- NDRG1, 2 and 4 inhibit proliferation and the epithelial-mesenchymal transition.

Abstract

Background:

The N-Myc Downstream-Regulated Gene (*NDRG*) family comprises four members that function in cellular processes like proliferation and differentiation. While *NDRG1* and *NDRG2* are extensively studied, knowledge regarding *NDRG3* and *NDRG4*, despite its recognition as a well-established early-detection marker for colorectal cancer (Cologuard[®]), is sparse.

Scope of review:

To summarize expression, biomarker potential and functional mechanisms of the *NDRGs* in the developing, mature and cancerous gut, we combine current literature and *in silico* analyses from the TCGA-database, GTEX Project, E14.5 mouse intestine and enteric neural crest cells, and an RNA-sequencing time-series of human embryonic colonic samples.

Major conclusions:

This study reveals that all members display a differential expression pattern in the gut and that *NDRG1*, *NDRG2* and *NDRG4* (1) can serve as biomarker for colorectal cancer and (2) have tumor suppressive properties mainly affecting cell proliferation and epithelial-mesenchymal transition.

General significance:

Similar effects of the *NDRGs* on the key-hallmarks of cancer, could implicate analogous functions in other tissue/cancer types.

Key words

Biomarkers; Colorectal cancer; Intestinal tract; N-myc downstream-regulated gene; Tumor suppressors

Introduction

The *N-myc downstream-regulated gene* (*NDRG*) family is composed of four members: *NDRG1*, *NDRG2*, *NDRG3* and *NDRG4*, and owes its name to the discovery of the first member, *NDRG1*, as being repressed by the *C-Myc* and *N-Myc* oncogenes [1]. In humans and mice, the four genes are all located on different chromosomes. Various aliases have been designated to each family member and each gene is transcribed into multiple alternatively spliced mRNA transcripts that encode several protein isoforms [2]. The encoded proteins of this family are all characterized by an NDR region and an α/β hydrolase fold, but lack the catalytic motif required to be enzymatically active [3, 4]. The *NDRG* proteins which share about 52-65% sequence homology with an identical C-terminal sequence “MEVSC” and only very few sequence differences primarily in the C- and N-terminal regions, have been shown to be highly conserved in a variety of species [5, 6]. More detailed information about the general knowledge on the *NDRG* family members, with respect to structure, origin and signaling in physiological processes can be found in our former review by Melotte et al. [2].

Previously, we and various independent groups identified *NDRG4* promoter CpG island methylation in fecal DNA as an accurate early-detection marker for colorectal cancer (CRC) [7-16]. The biomarker performance of *NDRG4* was further exploited by Exact Sciences, who integrated *NDRG4* promoter methylation into a multi-target stool DNA test: Cologuard® (Madison, USA) [16]. This FDA-approved stool DNA test detects significantly more CRCs and advanced precancerous lesions than the leading fecal immunochemical test (FIT) and is currently used in the USA for CRC screening [7]. Despite being incorporated into the Cologuard®, almost nothing is known about the function(s) of *NDRG4* in the (diseased) intestinal tract. Whereas

NDRG1 and NDRG2 are important in various cellular processes (e.g. proliferation, differentiation, apoptosis), little is known about NDRG3 and NDRG4.

Here, we performed a literature search (Pubmed, Web of Science, Embase and Medline) and supported this literature search with *in silico* data extracted from (i) publicly available transcriptome databases [17] [18], (ii) previously generated transcriptome data of E14.5 mouse intestine and enteric neural crest cells (ENCCs) [19] and (iii) an RNA-sequencing time-series of human embryonic colonic samples, to gain more insights into the expression pattern, biomarker potential and common pathways of the NDRGs in the intestinal tract .

Our review indicates that *NDRG1*, *NDRG2* and *NDRG4* can act as biomarkers for CRC. Although there is no evidence that the different family members are major drivers in the development of CRC, data suggest that, except for NDRG3, each NDRG member has a protective role during intestinal carcinogenesis.

Expression pattern of the NDRGs in the developing and adult gut

To understand the functional importance of the NDRG family members in the intestinal tract, we first elaborate on their structure and summarize their (overlapping) intestinal-specific expression pattern in mammalian species like rat, mouse and human.

In humans, *NDRG1* is located on chromosome 8q24.3, while the mouse ortholog is located on chromosome 15D2. The encoded canonical protein sequence of human NDRG1 (DRG1, RTP, RIT42, CAP43) consists of 394 amino acids (AA), resulting in a 43 kDa protein. The two other isoforms are made up of 328 and 313 AA, with a respective mass of 35.5 and 34 kDa. In mouse and rat, only one isoform has been found, with a similar length as the human canonical protein sequence and minor AA differences.

The expression of the *NDRG1* gene usually coincides in tissues where *N-myc* is expressed, but only initiates at E9.5 as the intestinal epithelium begins to differentiate and *N-myc* expression decreases [20]. As further intestinal development requires retinoic acid-dependent activation of *NDRG1* and suppression of the Wnt/ β -catenin pathway [21], *NDRG1* mRNA expression steadily increases in the mouse and human intestinal epithelium from E10.5 onwards [20]. Further intestinal maturation in rodents and humans seems to be controlled by circulating levels of glucocorticoids and cortisol, respectively [22], giving rise to similar levels of NDRG1 in the adult small and large intestine [5, 22-24]. Moreover, the expression of NDRG1 mRNA and protein is induced by glucocorticoid treatment (i.e. by dexamethasone) and strongly overlaps with a gradual increase from crypt cells towards surface cells [20, 22, 24, 25]. On a sub-cellular level, NDRG1 is primarily localized in the cytoplasm of epithelial cells, where it is often associated with the basolateral membrane adjacent to adherens junctions and desmosomes [20, 24, 26].

NDRG2 is located on chromosome 14q11,2 and 14C1 in human and mouse, respectively. The main functional form of human, mouse and rat NDRG2 (SYLD, NDR2) is either a 41 kDa protein, composed of 371 AA, or a 39 kDa protein of 357 AA. In addition, there are four other human isoforms with a length ranging from 328 to 367 AA and a variable mass between 36 and 40.3 kDa.

In early developmental stages (E8.5-9.5) the poorly differentiated intestinal epithelium expresses low levels of NDRG2 mRNA and protein. From E10.5 towards adulthood, the levels of NDRG2 mRNA and protein very slightly increase in the mucous forming, lining epithelial cells [20, 27], leading to barely detectable levels in adult gut tissue. Even though most studies observed a slightly higher level of *NDRG2* mRNA in the (distal) colon compared to the small intestine [5, 28, 29], contradictory observations regarding NDRG2 protein expression have been described.

Yamamoto et al. detected a marginally higher signal in colon than in the small intestine [30], whereas Hu et al. detected opposite results with weak to moderate NDRG2 immunoreactivity in the small intestine but not in the colon [31].

Human chromosome 20q11.21-11.32 and mouse chromosome 2H1 contain the *NDRG3* gene, which can be transcribed into three identified protein isoforms. The canonical sequence of *NDRG3* (no known aliases) encodes a 41.5 kDa protein of 375 AA in various mammalian species. The second and third isoforms are only found in humans and encode a 40 kDa protein of 363 AA and a markedly lighter protein (31.5 kDa) of only 286 AA.

Only three studies include a brief description about the expression of *NDRG3*. In fact, *in situ* mRNA hybridization (ISH) and Northern Blotting reveal that *NDRG3* expression is already activated at E9.5 and that *NDRG3* is widely expressed during embryogenesis. However no *NDRG3* mRNA is detected in the developing intestinal tract [20]. During further development, overall *NDRG3* mRNA and protein levels increase slightly, although they are hardly detectable in matured mouse and human gut tissues [5, 30].

The *NDRG4* gene is situated on human chromosome 16q21-q22.1 and mouse chromosome 8D1. For *NDRG4* (*SMAP8*, *BDM1*), the canonical protein sequence is referred to as *NDRG4B^{var}*, consisting of 352 AA (39 kDa). The two other main isoforms are *NDRG4B* and *NDRG4H* which contain 339 AA (37 kDa) and 371 AA (41 kDa), respectively. In addition, 5 other isoforms can be produced by alternative splicing, with sequences ranging from 339 to 391 AA (37-43 kDa). In mouse, we recently identified three isoforms, corresponding to human *NDRG4B^{var}* (long isoform), *NDRG4B* (short isoform) and *NDRG4H* [32]. Furthermore, rat tissues express an additional fourth isoform of 45kDa, encoded by the rat ortholog: *SMAP8/BDMI* [33].

So far, the pattern of NDRG4 expression during embryonic gut development has not yet been described. In adult tissues however, current literature is inconsistent regarding the intestinal-specific expression pattern of NDRG4 which can be attributed to the use of different, a-specific commercially-available antibodies [32]. We and Chu et al. previously described that NDRG4 is expressed within epithelial cells of the gut [12, 34] and Qu et al. found high NDRG4 protein levels in smooth muscle cells of the stomach [35]. However, we recently observed that the anti-NDRG4 antibody used in these studies was not specifically targeting NDRG4 [32]. The experimental application of antibody that specifically targets NDRG4 has demonstrated that NDRG4 expression in the gut is restricted to the cytoplasm of neuronal cell bodies and nerve fibers belonging to the nervous system of the gut, i.e. the enteric nervous system (ENS) [32].

In silico expression analysis

To clarify the discrepancies concerning the NDRG expression pattern in the developing/mature gut observed in the limited number of publications, we evaluated expression data from the (i) GTEx Project (06/19/2017), (ii) previously generated transcriptome data of E14.5 mouse intestine and ENCCs (available in the Gene Expression Omnibus (GEO); GSE34208) [19] and (iii) an RNA seq time-series of human embryonic full-thickness samples of proximal colon (Embryonic Week (EW) 12, EW14 and EW16; data generated by the group of Prof. Thapar (UCL, London) and deposited to GEO).

Compared to its family members, ***NDRG1*** is highly expressed in EW12, EW14 and EW16 human colonic samples, with levels (in reads per kilobase million, RPKM) being 10, 8 and 12 times higher than for *NDRG2*, *3* and *4*, respectively (Fig. 1A). In addition, *NDRG1* shows the highest and intercomparable expression in sections of the mature small intestine (Fig. 1B, terminal ileum) and colon (Fig. 1C-D, transverse and sigmoid). Using the RNA expression data set, we observed

that *NDRG2* is expressed in low levels in human embryonic intestine, similar as described by Okuda et al. and Hu et al. [20, 27] (Fig. 1A). In contrast to *NDRG1*, little *NDRG2* mRNA is detectable in the adult human intestinal tract, which is only marginally different in small intestinal and colonic tissues (Fig. 1B-D). These very low expression levels may account for the in-literature-described inconsistencies of intestinal *NDRG2* protein expression being either increased or decreased in colon versus small intestine.

Similar as observed for *NDRG2*, little *NDRG3* mRNA is detectable in the developing and mature human intestinal tract. In fact, *NDRG3* mRNA levels in embryonic and adult human intestines are about eight and seven-fold lower compared to *NDRG1* (Fig. 1A).

While no literature describes the pattern of *NDRG4* expression during intestinal development, our analysis shows that of the four members, *NDRG4* has the lowest mRNA level in human embryonic gut tissues. In humans, *NDRG4* expression in the embryonic bowel is approximately twelve times lower compared to *NDRG1* (Fig. 1A). Consequently, only low to intermediate levels of *NDRG4* can be detected in whole-gut tissues of adult humans (Fig. 1C). Interestingly, as we recently observed that *NDRG4* is exclusively located in structures of the ENS [32], we wondered whether *NDRG4* expression in early developmental stages is also found in structures that will form the ENS, i.e. the enteric neural crest cells (ENCCs). Therefore, we compared the expression of the *NDRGs* in whole-gut biopsies with ENCCs, which we previously isolated from E14.5 mouse intestine [19]. Corresponding with its ENS-specific expression in adult gut, *NDRG4* mRNA is significantly higher expressed in ENCCs compared to total gut tissue of E14.5 mouse embryos (Fig. 1E; 15.88 fold change, FDR < 1e-07). Thus, it can be stated that *NDRG4* is continuously expressed by ENCCs upon colonization of the gut and development into the ENS upon intestinal maturation, which accounts for the low level of expression in whole-gut tissues of adult mice/humans. This raised the question if the other family members are also

expressed uninterruptedly within the epithelial cells or ENCCs throughout development. When comparing the expression analyses of whole-gut biopsies and ENCCs, we observed that *NDRG1* is hardly detectable in ENCCs, but highly expressed in embryonic and mature mouse and human total gut samples (Fig. 1A, E; fold change 0.11, FDR < 1e-07; Fig. 1B-D) [19]. These data, combined with the in literature-described expression of *NDRG1* in the (developing) epithelial layer, suggests that *NDRG1* remains mainly expressed within the epithelium throughout intestinal maturation. On the other hand, the expression of *NDRG2* and *NDRG3* is respectively 3.0 and 2.57-fold higher in ENCCs compared to total gut tissue of E14.5 mouse embryos (Fig. 1E; FDR *NDRG2* = 1.08e-05 and FDR *NDRG3* < 1e-07). Thus, besides valid concerns on whether different anti-*NDRG2/3* antibodies or experimental procedures are specific enough for the identification of *NDRG2/3* in adult gut, it is also possible that the expression of *NDRG2/3* shifts towards the epithelial cells upon further intestinal maturation. Hence, further investigation is needed to unravel if *NDRG2* and *NDRG3* expression is retained in ENCCs and appears in the ENS or rather moves to the epithelial cells during gut development.

Differential expression levels during intestinal carcinogenesis

Multiple studies have investigated changes in *NDRG* mRNA and protein expression in neoplastic (adenomas, primary tumors, metastatic lesions, intestinal cancer cell lines) compared to normal tissue, which we further explored using the publicly available TCGA data. Moreover, we used current literature and TCGA data to identify possible mechanisms that regulate the differential expression of the *NDRGs* during intestinal carcinogenesis.

Differential expression levels

While the limited knowledge on **NDRG3** in the healthy gut hinders further evaluation of NDRG3 expression during intestinal diseases, several studies discuss the differential expression of NDRG1, NDRG2 and NDRG4 during colorectal carcinogenesis. However, studies that described changes in NDRG4 protein levels are based on the inaccurate detection of NDRG4 (as described above [12, 34, 36]) and are thus not further taken into account here.

Only four studies described normal or increased levels (i.e. preserved levels) of *NDRG1* expression in tumor tissues compared to normal tissues [37-40]. Nevertheless, six studies agree on significantly reduced mRNA and protein levels of **NDRG1** [23, 26, 40-43], but also **NDRG2** [44-50], and **NDRG4** [12, 51] in neoplastic tissues compared to healthy colonic epithelium. Moreover, mRNA and protein levels of both NDRG1 and NDRG2 decrease with increasing tumor grade: NDRG1 and NDRG2 are highly expressed in normal and low-risk adenoma tissue and show a gradual decrease from well- to moderately to poorly or undifferentiated tumor tissue and to metastatic lesions [23, 26, 41, 43-50]. These findings are further supported by several *in vitro* studies demonstrating (i) the high expression of *NDRG1* in the normal colon epithelial cell line NCM460 and the low expression of *NDRG1* and *NDRG4* in several colon cancer cell lines (e.g. HCT116, SW480, RKO) [12, 52], (ii) the higher level of NDRG1 and NDRG2 in primary colon cancer cell lines (e.g. HCT116 and SW480) compared to metastatic colon cancer cell lines (e.g. SW620, Lovo) [23, 46, 53], (iii) the up-regulation of both *NDRG1* and *NDRG2* upon differentiation (e.g. by N-acetyl-L-cysteine) of primary colon cancer cell lines (e.g. HT29) (23, 26, 55, 60), and (iv) the induction of morphological changes and increased expression of important epithelial cell differentiation markers by *NDRG1* overexpression in metastatic colon cancer cells [23, 54].

Using data from the publicly available TCGA database containing 261 colorectal cancer patients and 41 colonic healthy controls we further investigated the expression of all four *NDRG* members in cancerous compared to healthy colon tissue (Fig. 2A). While no literature describes

NDRG3 expression during colorectal carcinogenesis, the TCGA data reveal that the expression is only marginally, but not significantly up-regulated in CRC tumors compared to normal colon (Fig 2A). Moreover, in agreement with most literature studies, the level of *NDRG1*, *NDRG2* and *NDRG4* mRNA is significantly reduced in colorectal tumor tissue compared to normal colonic tissue (Fig. 2A; Wilcoxon rank sum test, all $P < 0.001$). Consequently, it is thus extremely likely that NDRG1 protein levels will also be reduced during CRC and that above described discrepancies (i.e. lower vs. higher expression in tumor tissue) can be attributed to the usage of different (non-) commercially available anti-NDRG1 antibodies or variations in experimental procedures, yet are most presumably explained by the highly variable outcome of NDRG1 positivity measurements, using different variations of a semi-quantitative measurement method (i.e. a calculation of NDRG1-positive cells per area (%) combined with a score reflecting the intensity of NDRG1 immunoreactivity (0-3)).

Causes of NDRG loss

Genetic changes

Only sporadically, mutations for *NDRG* family members have been identified. So far no studies have investigated whether *NDRG1* is mutated during colorectal tumorigenesis, while it is well-described that *NDRG1* mutations lead to hereditary motor and sensory neuropathy-Lom (HMSNL), a severe autosomal recessive peripheral neuropathy (Charcot-Marie-Tooth disease) [55]. Further, there is no evidence for (in)activating mutations of *NDRG2*, *NDRG3* and *NDRG4* throughout the course of CRC in current literature, and the single-nucleotide polymorphisms (SNPs) that affect *NDRG4* in 75-100% of primary colorectal cancers, do not alter its AA sequence [12]. Similarly, we found little evidence for cancer-related mutations in any of the *NDRG* genes in the publicly

available TCGA mutation data: *NDRG1*: 2/154 (missense mutation and silent SNP), *NDRG2*: 6/154 (2 missense mutations and 4 silent SNPs), *NDRG3*: 1/154 (missense mutation) and *NDRG4*: 2/154 (intron SNP and frame shift mutation).

Chromosomal imbalances have also been shown to play a role in the pathogenesis of stromal tumors of the gastrointestinal (GI) tract. During colorectal carcinogenesis no chromosomal disparities containing *NDRG1* have been described. Although Ässämäki et al. and Lorentzen et al. have identified that copy number changes might affect *NDRG2* in tumors of the GI tract, the first study shows that *NDRG2* is the most commonly deleted region at 14q11.2 in GI tumors [56], whereas the latter recently revealed that allelic loss of *NDRG2* is less frequent than a copy number gain in CRC [57]. For *NDRG4*, we previously observed loss of heterozygosity in 27% of CRCs [12]. As described above, alterations in *NDRG3* have not been examined during intestinal diseases in literature. Nonetheless, analysis of the TCGA copy number data (Fig. 2B) revealed a significant copy number gain for *NDRG1* and *NDRG3* in tumor compared to normal samples ($P < 0.001$) and a small, but significant decrease in copy number for *NDRG2* ($P < 0.001$). For *NDRG4*, no change in copy number was observed using the TCGA data (Fig. 2B).

Epigenetic changes

The presence of multiple CpG islands in the promoter region of *NDRG1*, *NDRG2* and *NDRG4* renders these genes suitable for regulation by DNA methylation during carcinogenesis. In line, primary and metastatic colon cancer cell lines (e.g. SW480 and SW620) show minimal *NDRG1* CpG island methylation [53] and a number of GI-cancer cell lines and colon cancer tissues show variable *NDRG2* promoter hypermethylation at several CpG sites, with levels ranging between 27 to 100% [45, 57-60]. Promoter hypermethylation of *NDRG2* corresponds well with reduced *NDRG2* expression in CRC [45, 57-60]. In fact, 84-100% of the hypermethylated tissues lack

NDRG2 mRNA expression and immunoreactivity [57-59]. The differential methylation pattern could be explained by the presence of several transcription start sites (TSS) with distinct promoters having CpG islands, primarily located in exon 1 and 2 of the *NDRG2* gene [50], and the use of different primer sequences to analyze the methylation status of the *NDRG2* promoter [45, 50, 58, 59, 61]. Similarly, for *NDRG4* promoter methylation, we showed that a primer pair located in the region with dense promoter methylation (more 5' relative to TSS) yielded a sensitivity of 86% for CRC detection, which decreased to 71% when using a second primer pair located in a region with less dense promoter methylation (more 3' relative to TSS) [12].

The epigenetic regulation of *NDRG1*, *NDRG2* and *NDRG4* during CRC has been further confirmed by the reduced promoter methylation and consequential increase in *NDRG1*, *NDRG2* and *NDRG4* expression in GI-cancer cell lines treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytine (DAC). Moreover, treatment with the histone deacetylase inhibitor trichostatin A (TSA) further enhanced the expression of *NDRG1*, *NDRG2* and *NDRG4*, suggesting that their expression is regulated by histone acetylation and promoter methylation [12, 23, 45, 50, 53, 58-61]. In contrast, the mechanism of epigenetic silencing of *NDRG3* expression has, to our knowledge, not been described in literature.

TCGA methylation data for 272 colorectal cancer tissues and 37 colonic tissues from healthy controls (Fig. 2C), confirm the minimal CpG island methylation of *NDRG1*, as only four out of ten Infinium 450k probes within 2 kb either side of the TSS of *NDRG1* showed a significant difference in methylation between tumor and normal samples (Wilcoxon rank sum test, $P < 0.001$). In addition, TCGA data show a variable degree of methylation at different CpG sites in *NDRG2*, with 1/14 and 7/14 of the analyzed CpGs, respectively, having a significantly reduced or increased methylation status in CRC (Wilcoxon rank sum test, $P < 0.001$). Further, in tumor tissue of CRC patients, the *NDRG4* gene is characterized by the highly increased methylation of 13/15 promoter

CpG sites compared to healthy controls (Wilcoxon rank sum test, $P < 0.001$). Finally, TCGA-derived methylation data show a statistically significant increase in methylation for four out of seven promoter CpGs of the *NDRG3* gene when comparing CRC to normal samples (Wilcoxon rank sum test, $P < 0.001$). The observed methylation is however, unlikely to have any biological effect as *NDRG3* expression between tumor and normal samples is not changed (Fig. 2A).

In addition, chromatin remodeling and miRNAs can also influence the gene expression of *NDRG1* and *NDRG2* in the development and progression of CRC. For instance, the chromatin structure of *NDRG1* in SW480 cells has been described to be more accessible for the Polymerase II enzyme, suggesting a more active transcription of *NDRG1* and a higher level of *NDRG1* expression in SW480 compared to SW620 cells [23, 53]. According to Feng et al. *NDRG2* represents a direct target of microRNA-650 (miR-650): increased miR-650 levels diminish *NDRG2* expression in colon cancer cells, which is reversible by treatment with anti-miR-650 [45].

The biomarker potential of the NDRG members

Due to their differential expression in various conditions, numerous studies have investigated the correlation between the level of *NDRG1*, *NDRG2* and *NDRG4*, and various clinico-pathological variables and studied their potential as diagnostic, prognostic or predictive biomarkers for CRC (Suppl. table 1).

Diagnostic biomarkers

NDRG4 is the only NDRG-relative that has been studied in diagnostic biomarker research owing to its distinct level of DNA (promoter) methylation in healthy people versus CRC patients.

In 2009, we reported that the frequency of *NDRG4* DNA promoter methylation significantly increased from normal (0-16%) to adenoma (61-63%) to carcinoma tissue (84-86%) [12]. Several independent groups (Suppl. Table 1) confirmed that *NDRG4* DNA promoter methylation could sensitively discriminate CRC tissues (68-76%), and advanced adenomas (81.0-89.0%) from normal mucosa, with a specificity of 91.7-97.0% [8, 13, 62, 63]. Although most studies conclude that the frequency of *NDRG4* DNA promoter methylation in tissues is not associated with any clinicopathological variable, Zou et al. and Park et al. described that the *NDRG4* promoter methylation significantly increased with age during carcinogenesis ($P < 0.001$), and that methylation tends to be higher in patients aged ≥ 60 ($P = 0.077$) [62, 63]. Besides, the frequency of *NDRG4* promoter methylation has been shown to be higher in (i) right versus left colon tumors ($P < 0.001$ -0.290), (ii) stage III and IV CRC compared to stage I and II CRCs and (iii) in females with FIT-negative versus females with FIT-positive cancers ($P = 0.003$) [62, 64].

To investigate the utility of *NDRG4* DNA promoter methylation as a noninvasive biomarker for the detection of CRC, several studies examined the frequency of *NDRG4* DNA promoter methylation in fecal DNA of CRC patients and healthy controls. We and numerous independent groups revealed that *NDRG4* DNA promoter methylation in fecal DNA, as a single marker has a sensitivity for the detection of CRC patients between 29-88% at a high specificity (89-100%) [7-15].

Notwithstanding the performance of *NDRG4* DNA promoter methylation as a single stool marker, several studies combined *NDRG4* with other markers to investigate if this would enhance the sensitivity and specificity for CRC detection. The multi-target stool DNA test that combines several methylated markers (e.g. *NDRG4*, *Vimentin*, *BMP3*, *TFPI2*), with mutant *KRAS* and/or fecal hemoglobin, can detect 85.0-87.0% of CRCs, 42.4-92.0% of CRC + adenomas + dysplasia, and 54.0% of adenomas ≥ 1 cm at 87.0-94.0% specificity [7, 65-68]. Interestingly, the combination

of only *NDRG4* and *BMP3* DNA promoter methylation further enhanced the sensitivity to 100% at 89% specificity [10]. Consequently, Exact Sciences (Madison, USA) developed the Cologuard® test that combines a marker panel of methylated *NDRG4* and *BMP3*, mutant *KRAS*, and a quantitative detection of fecal hemoglobin for an even more accurate detection of CRC. The accurate performance of the Cologuard® for CRC screening was highlighted by a number of studies and further established by Imperiale et al. in a large multi-center trial including over 10.000 participants at average risk for CRC. Data from these studies revealed that the Cologuard® has a sensitivity of 92.3-100%, for the detection of CRC patients and of 42.4% for the detection of advanced precursor lesions, at a specificity of 86.6-91.0%, irrespective of stage, size and tumor location [14, 16, 66, 69]. Finally, similar as observed for methylated *NDRG4* alone, the sensitivity of both the stool DNA test and Cologuard® typically increased with adenoma and CRC size [7, 65, 69].

At last, it has been described that *NDRG4* DNA promoter methylation in plasma has a sensitivity of 27.0%- 54.8% to detect CRC patients at a specificity of 78.1-95.0% [13, 70] and that *NDRG4* DNA promoter methylation in urine samples also sensitively (72.6%) detects CRC, yet at a slightly reduced specificity (85.0%) [13].

Prognostic biomarkers

As described above, the mRNA and protein levels of **NDRG1** in tumor tissue significantly differ from the levels observed in normal gut tissue. All studies agree that the level of NDRG1 mRNA or protein expression is not influenced by age and gender, nor with the localization of the primary tumor (Suppl. Table 1). Nevertheless, except for three studies [38-40], all studies observed that *NDRG1* expression is inversely correlated with the degree of lymph node and distant metastasis and advanced TNM stage, while being positively associated with well-differentiated (less

aggressive) tumors. Moreover, these studies found *NDRG1* to be an independent, favorable prognostic factor for CRC. In fact, when not differentiating between cancer stages, the overall survival (OS) rate for CRC patients with *NDRG1* expression was remarkably higher compared to those with reduced *NDRG1* levels ($P=0.000-0.148$) [40, 42, 43, 54]. When looking at separate stages, the survival rate of stage II and IV patients with preserved *NDRG1* mRNA expression in the tumor was significantly better compared to patients with reduced *NDRG1* tumor levels ($P=0.0005$) [40]. In addition, the disease free survival (DFS) and progression free survival (PFS) rate were 1.3-2.0 fold higher for the patients with preserved *NDRG1* protein expression in the tumor compared to the ones having reduced *NDRG1* tumor levels ($P=0.003-0.004$) [42, 54], suggesting that *NDRG1* is an independent prognostic factor for both recurrence and overall survival. Interestingly, Koshiji et al. reported that *NDRG1* expression significantly correlated with Duke's stage in Japanese ($P=0.036$), and Caucasians ($P=0.0063$), but not in African Americans ($P=0.880$). In addition, a worse survival rate was observed in US African American patients only ($P=0.035$), suggesting that the ethnic background affects the level of *NDRG1* expression and its correlation with clinico-pathological variables and disease outcome [38].

While different studies investigated the correlation of **NDRG2** expression with clinico-pathological variables, only two further explored the prognostic potential of *NDRG2* [44, 58]. *NDRG2* mRNA and protein levels have been described to positively correlate with the differentiation state of the tumor ($P<0.0001-0.450$) and inversely relate to TNM staging ($P<0.001-0.772$) and lymph node metastasis ($P<0.001-0.214$). In addition, Hong et al. reported a higher level of *NDRG2* promoter methylation in the proximal compared to the distal colon ($P=0.022$) and in advanced T-stage tumors ($P=0.039$) [58]. No significant association was observed between *NDRG2* expression and age, gender or distant metastasis. However, CRC patients with tumors characterized by high levels of *NDRG2* promoter methylation or reduced expression of *NDRG2*

have a worse OS rate ($P=0.003-0.145$) as well as a shorter DFS/higher recurrence risk ($P=0.002-0.216$) compared to those having tumors with preserved *NDRG2* expression or low levels of *NDRG2* promoter methylation [44, 58]. Consequently, *NDRG2* also has the potential of being a prognostic biomarker for CRC.

While we have to be cautious with the conclusions drawn in the study by Chu et al. regarding the prognostic potential of **NDRG4** [34], as these were based on the inaccurate detection of *NDRG4* with a non-specific anti-*NDRG4* antibody [32], a second study by this group (Zheng et al.) shows similar results using differential mRNA expression analysis [51]. The OS and DSF time of patients with *NDRG4* mRNA positive tumors was significantly improved compared to the group with *NDRG4* negative tumors ($P<0.001$ for both) [51]. Furthermore, *NDRG4* expression was not affected by age, gender or tumor location, whereas it was significantly positively correlated with tumor differentiation ($P=0.001$), and inversely related to distant ($P=0.001$) and lymph node metastasis ($P=0.004$) and TNM staging ($P=0.001$) [51].

Predictive biomarkers

Only a very limited number of studies have investigated the predictive value of **NDRG1** in CRC cell lines and CRC patients [43, 54]. Wangpu et al. observed an increased sensitivity of *NDRG1*-overexpressing HT29 and HCT116 cells to increasing concentrations of 5-fluorouracil ($P<0.001-0.010$) [71]. Ma et al. described that SW480 cells are more prone to Irinotecan treatment upon knockdown of *NDRG1*, with a significantly lower IC_{50} value compared to control transfected cells ($P<0.050$) [54]. Similarly, a high level of *NDRG1* expression tends to cause resistance to Irinotecan-based therapy, as patients who developed a recurrence while having high *NDRG1* tumor levels, received Irinotecan-based therapy only for 6.8 months, whereas those with a low tumor level

of NDRG1 remained on this therapy for 9.3 months (P=0.070) [43]. No studies have explored the predictive biomarker value for either *NDRG2*, 3 or 4.

Functional roles and pathways involving the NDRGs

It has been recognized that the NDRG family mainly functions in proliferation and differentiation in multiple types of cells/cancers (for reviews we refer to [72-75]). The limited number of studies that suggest a regulatory function for NDRG3 and NDRG4 in proliferation and differentiation are summarized by Yang et al. [76] and Melotte et al. [2]. In this review, we further elaborate on the mechanisms that have consequences for intestinal functions in normal and cancerous conditions.

Cell survival: proliferation versus p53/apoptosis

Kim et al. observed that **NDRG1** is localized in the centrosome of CRC cells as a microtubule-associated protein and participates in the cell cycle as a mitotic checkpoint [77]. Furthermore, in p53-deficient tumor cell lines (incl. the CRC cell lines DLD-1 and HCT116-*p53*^{-/-}), NDRG1 was shown to inhibit polyploidy development and to increase the number of cells in cell cycle arrest [77]. Hence, NDRG1 can protect against uncontrolled proliferation. Stein et al. further reported that NDRG1 expression was induced by DNA damage through a mechanism involving p53 in these CRC cell lines [78], which could explain the increased level of NDRG1 in response to hypoxia, as hypoxia induces p53 accumulation and stabilization [78]. Later, Zhang et al confirmed that another type of stress, nutrient/polyamine depletion, can stabilize p53 and by this means enhance NDRG1 expression in normal intestinal epithelial (IEC-6) and CRC cells (HCT116-*p53*^{-/-}) [79]. In both studies, NDRG1 expression was not sufficient to induce apoptosis, but it did inhibit DNA synthesis and proliferation [78, 79]. In contrast, Ambrosini et al. revealed that the shRNA-mediated silencing

of *NDRG1* in HCT116 cells led to increased levels of the pro-apoptotic Bim protein [80], similar as reported by Ma et al. [54]. When *NDRG1* was reintroduced in the cells, it suppressed Bim stability by mediating its binding to the proteasome, thereby repressing the apoptotic machinery [80]. Thus, NDRG1 likely functions in controlling and repressing proliferation, but is not sufficient to induce apoptosis in intestinal cells.

Several studies suggest the anti-proliferative effects of **NDRG2** in CRC cells (e.g. SW48, SW480, SW620, DLD-1, HCT116). Kim et al. first showed that NDRG2 suppresses AP-1, which results in the down-regulation of cyclin D1, the subsequent cell cycle arrest in the G1/S phase and a concomitant reduction in cell proliferation [46, 81]. Further, Golestan et al. and Hong et al. observed that the overexpression of *NDRG2* in SW48, DLD-1 and RKO cells reduces cell viability and inhibits cell proliferation, suggesting that NDRG2 might have tumor suppressing roles [58, 82].

Likewise, but not restricted to CRC cells, Park et al. observed that hypoxia induced the accumulation of **NDRG3** and the subsequent activation of the Raf-ERK pathway, which was associated with increased anti-apoptotic effects [83].

Previously, we observed that *NDRG4* overexpression in CRC cells suppressed colony formation and the proliferative capabilities of these cells [12], suggesting a tumor suppressor role in CRC. In addition, Chu et al. proposed further evidence for the anti-proliferative effects of NDRG4 during CRC, as they described how *NDRG4* overexpression in the metastatic SW620 CRC cell line considerably decreased PI3K-AKT activation by reducing AKT phosphorylation [12, 34]. No other studies examined potential pathways of NDRG4 in CRC.

Metastasis/invasion/epithelial-mesenchymal transition

As described-above, metastatic CRC tissue and cell lines express reduced levels of endogenous ***NDRG1***. In view of this, several studies have shown that NDRG1 is able to suppress metastasis and invasion. Guan et al. found that *NDRG1* overexpression in the metastatic CRC cell line SW620 inhibits invasion [23]. Moreover, even after treatment with TGF- β [71, 84], NDRG1 suppresses the epithelial-mesenchymal transition (EMT) in colon cancer cells (e.g. HCT116, HT29) by preventing stress fiber formation and cell motility through suppression of the ROCK1/pMLC2 pathway and by stimulating the membrane expression of E-cadherin and β -catenin [85]. More precisely, NDRG1 induced three mechanisms to maintain β -catenin at the cell membrane by: (i) inhibiting the phosphorylation of β -catenin through up-regulating the GSK-3 β -binding protein, frequently rearranged in advanced T-cell lymphoma 1 (*FRAT-1*), and (ii) preventing the nuclear localization of β -catenin via down-regulation of *p21* activated kinase 4 (*PAK-4*) distribution in the nucleus [71, 86] and (iii) ensuring the ubiquitylation and subsequent degradation of caveolin-1 (*cav-1*) [87]. Similarly, NDRG1 prevents the EMT and metastatic/invading capabilities of CRC cells by inhibiting NF- κ B (a key player in oncogenesis) and Cav-1: as *NDRG1* knockdown increased the levels of NF- κ B, Cav-1 and the mesenchymal markers Vimentin and Snail, while decreasing the expression of the epithelial marker E-cadherin [54, 87]. Finally, NDRG1 prevents the recruitment of a key proto-oncogene: cellular Src to EGFR, thereby inhibiting Src phosphorylation (Tyr416) and its subsequent activation, thus inhibiting metastasis [88]. In addition, overexpression of *NDRG1* in HT29 cells reduced cell migration, focal adhesions, and cell collagen adhesion through a diminished activation of the important oncogenic FAK/paxillin pathway [89].

Similar as for NDRG1 [84], Shen et al. investigated the effect of ***NDRG2*** on TGF- β -treated HT29 cells [90]. TGF- β , which has tumor suppressor activities in early CRC stages but oncogenic actions in late stages, enhanced the EMT through down-regulation of E-cadherin and up-regulation of

Vimentin. Importantly, overexpression of *NDRG2* could reverse this TGF- β -induced EMT transition whereby cells restored their epithelial morphology, while *NDRG2* knockdown further enhanced the TGF- β -induced EMT [90]. In addition, *NDRG2* exerts its suppressive effects on invasion and migration in DLD-1, RKO and SW48 cells probably through inhibition of matrix metalloprotease (MMP)-9 activity, which is involved in tumor invasion via degradation of extracellular matrix proteins and activation of TGF- β [58, 82].

In accordance with the effects of *NDRG1* on β -catenin, *NDRG2* has been shown to (in-) directly reduce β -catenin and its major downstream T-cell factor/lymphoid enhancer factor (TCF/LEF) signaling pathway in various CRC cell lines (e.g. HCT116 and SW620 cells). Hwang et al. revealed the direct association of the *NDRG2* protein, with β -catenin and the subsequent inhibition of TCF/LEF signaling in SW620 cells [91]. On the other hand, *NDRG2* can activate GSK-3 β , which in turn induces the degradation of β -catenin and subsequently suppresses *C-Myc* [92, 93]. This *NDRG2*-mediated activation of GSK-3 β also induced degradation of Snail, followed by de-repression of E-cadherin [46, 94]. Together, these effects led to increased levels of E-cadherin and β -catenin at the plasma membrane, thereby inhibiting the EMT. Lastly, Kim et al. determined the prenylated Rab acceptor 1 (PRA1), a protein involved in vesicle trafficking and protein transport, to be a binding partner for *NDRG2* [95]. They synergistically inhibit GSK-3 β phosphorylation, thereby preventing nuclear translocation of β -catenin. Interestingly, *NDRG1*, *NDRG2*, and *NDRG3* all suppress cell growth and TCF promoter activity by their synergistical interaction with PRA1 [95].

We previously described that ***NDRG4*** inhibits metastasis, by reducing the invading capacities of *NDRG4*-overexpressing HCT116 cells [12], most likely by targeting some of the above-mentioned pathways. Indeed, Chu et al. observed that *NDRG4* suppresses PI3K-AKT activity, since *NDRG4* overexpression in SW620 cells reduced AKT phosphorylation [34]. As

AKT is a well-known upstream inhibitor of GSK-3 β , we postulate that upon *NDRG4* expression, the inhibition of GSK-3 β is abolished through down-regulation of AKT, thereby leading to degradation of β -catenin and *C-Myc* repression, resulting in the inhibition of the EMT. Although in the heart, Xing et al. observed a similar mechanism during ischemia-reperfusion injury, where the TNF- α -dependent NF- κ B activation increased myocardial expression of *NDRG4*, which is associated with a concomitant decrease in AKT phosphorylation, promoting apoptosis [96].

A schematic overview of the above-described pathways influenced by *NDRG1*, *NDRG2*, *NDRG3* and *NDRG4* during cell survival and the EMT are shown in figure 3A and B, respectively.

Conclusion

Overall, we can deduce that the *NDRGs* have a variable expression pattern, both in the embryonic and adult gut. *NDRG1* expression highly exceeds the expression of *NDRG2*, *NDRG3* and *NDRG4* in the fetal gut. Although one might argue this is possibly caused by a lower expression or less specific cell types expressing *NDRG 2, 3* and *4* in total gut biopsies, we observe a similar pattern in the matured GI-tract, suggesting that the intestinal epithelium is rich in *NDRG1*. *NDRG2, 3* and *4* are however, up-regulated in ENCCs during embryonic stages, but display a distinct pattern in the mature gut: *NDRG2* expression in epithelial cells, an unknown pattern for *NDRG3* and the ENS-specific expression of *NDRG4*. Hence, further studies should be performed to resolve the fundamental question whether expression of *NDRG2* and *3* is retained in ENCCs during intestinal maturation and appears in the ENS of the adult gut or rather moves towards the cells of the epithelial layer during gut development.

Furthermore, the above-described knowledge regarding the developmental expression of *NDRG2*, *NDRG3* and *NDRG4* in ENCCs, and the adult expression of *NDRG4* specifically in the ENS, led to the question whether the *NDRGs*, via the ENS, contribute to the development and progression of CRC. While it is well-known that the ENS is pivotal to maintain gut homeostasis, there is little evidence linking the ENS with CRC. In our recent review by Rademakers et al. we summarized the preliminary evidence linking the ENS with CRC and hypothesized that enteric neurons communicate with the intestinal epithelium via the production and secretion of neurotrophic factors through vesicle transport [97]. Interestingly, *NDRG4* has been shown to regulate some important SNARE-proteins of the vesicle trafficking machinery as (i) its interaction with *BVES* regulates docking and cargo delivery of *VAMP-3* vesicles, and (ii) its knockdown is associated with a sharp reduction in the level of *SNAP-25*. Moreover, we here review the interaction of *NDRG2* (*NDRG1* and *NDRG3*) with *PRA1*, a protein involved in exocytosis by binding to the SNARE-receptor. Finally, *NDRG3* interacts with *CPLX1*, which regulates synaptic vesicle exocytosis by allowing SNAREs (like *SNAP25*) to release neurotransmitters only upon arrival of an action potential [98]. Thus we hypothesize that the *NDRGs* in the ENS can affect the intestinal epithelium through regulation of vesicle transport.

Besides, we acknowledge the significance of the differential expression of *NDRG1*, *NDRG2*, *NDRG3* and *NDRG4* in normal and cancerous intestinal tissues, which is, for *NDRG2* and *NDRG4* mainly caused by DNA promoter hypermethylation. Additionally, the increased expression of *NDRG1* and *NDRG2* during intestinal epithelial cell differentiation and reduced expression of *NDRG1*, 2 and 4 in the cancerous gut and their metastasis, suggest a possible role for these family members as tumor/metastasis suppressor genes.

Based on their differential expression patterns, the *NDRGs* are described as potential diagnostic (*NDRG4*), prognostic (*NDRG1*, 2 and 4) and predictive (*NDRG1*) biomarkers for CRC.

Indeed, the highly up-regulated level of *NDRG4* DNA promoter methylation in fecal DNA represents a non-invasive early detection marker for CRC that has been successfully implemented in clinical use in the USA, as part of the Cologuard® test. While the significantly reduced expression of *NDRG1*, 2 and 4 in tumor tissues of CRC patients can define the prognostic value for CRC (i.e. worse OS and DSF), it is important to realize that these levels may vary when using different antibodies, varying experimental techniques, and even in population groups with a diverse ethnic background. Furthermore, the potential predictive value of *NDRG1* for response to Irinotecan or 5-Fluorouracil treatment in CRC needs more in-depth investigation before it can be applied in the clinic.

Finally, we reiterate that *NDRG1*, 2 and 4 exert pleiotropic effects, mostly inhibiting key hallmarks of carcinogenesis (e.g. proliferation, differentiation, metastasis) during CRC. Indeed, *NDRG1* and *NDRG2* have been shown to alter proliferative and apoptotic pathways (e.g. cell cycle and EGFR signaling) and the epithelial-mesenchymal transition. Limited data on the functional role of *NDRG4* in the gut also suggest tumor suppressor activities for *NDRG4* during CRC, whereas no mechanism of action for *NDRG3* in colorectal carcinogenesis has been defined. More insight into the pathways in which *NDRG3* and *NDRG4* are involved, can be drawn from described effects in other (cancerous) tissue types and suggests that *NDRG3* affects the apoptotic machinery [83] and that *NDRG4* is able to modulate similar targets as defined for *NDRG1* and *NDRG2*, in apoptotic, proliferative (e.g. *p53*, *cyclin D1*) [99-101] and EMT-signaling pathways (e.g. WNT/ β -catenin, TGF- β , EGFR-downstream targets) [34, 102, 103]. Nevertheless, it is important to keep in mind that despite the involvement of *NDRGs* in similar pathways, their effects may vary (i.e. tumor suppressor versus oncogene) depending on the tissue type.

Figures & figure captions

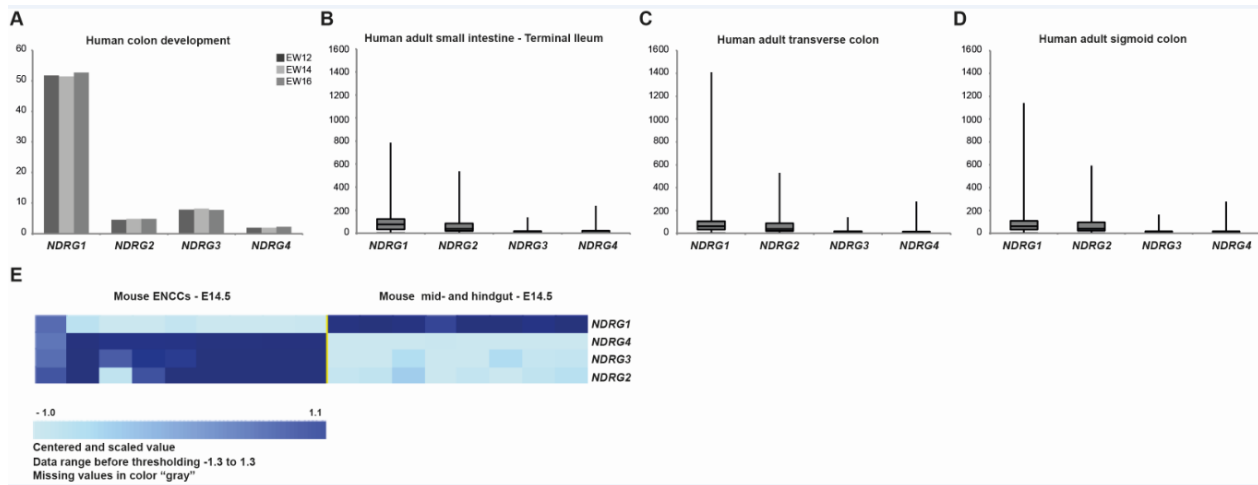


Figure 1. *The expression profile of the NDRG family members in humans and mice. A.* RNA expression analysis of the *NDRGs* during human intestinal development shows that *NDRG1* is highly expressed during human colon development (Embryonic Week (EW) 12, 14 and 16), being about 10, 8 and 12 times higher than *NDRG2*, 3 and 4, respectively. RNA was taken from embryonic full-thickness samples of proximal colon; boxplots represent measurements of one sample per developmental time-point in triplo in reads per kilobase million (RPKM); data submitted to GEO. **B-D.** In human adulthood, *NDRG1* expression is highly variable, yet exceeds that of *NDRG2*, 3 and 4 in the small intestine (B) and colon (C, transverse; D, sigmoid). The data used for the analyses were obtained from the GTEx Portal on 06/19/2017. **E.** In gut-associated cells from E14.5 mouse embryos, *NDRG2*, 3 and 4 expression (measured with micro-array) is significantly up-regulated in Enteric Neural Crest Cells (ENCCs; *NDRG2*: 3.0-fold change, FDR = 1.08e-05; *NDRG3*, 2.6-fold change, FDR < 1e-07; and *NDRG4*, 15.9-fold change, FDR < 1e-07), while *NDRG1* is highly expressed and slightly up-regulated in the total gut (0.11-fold change, FDR < 1e-07).

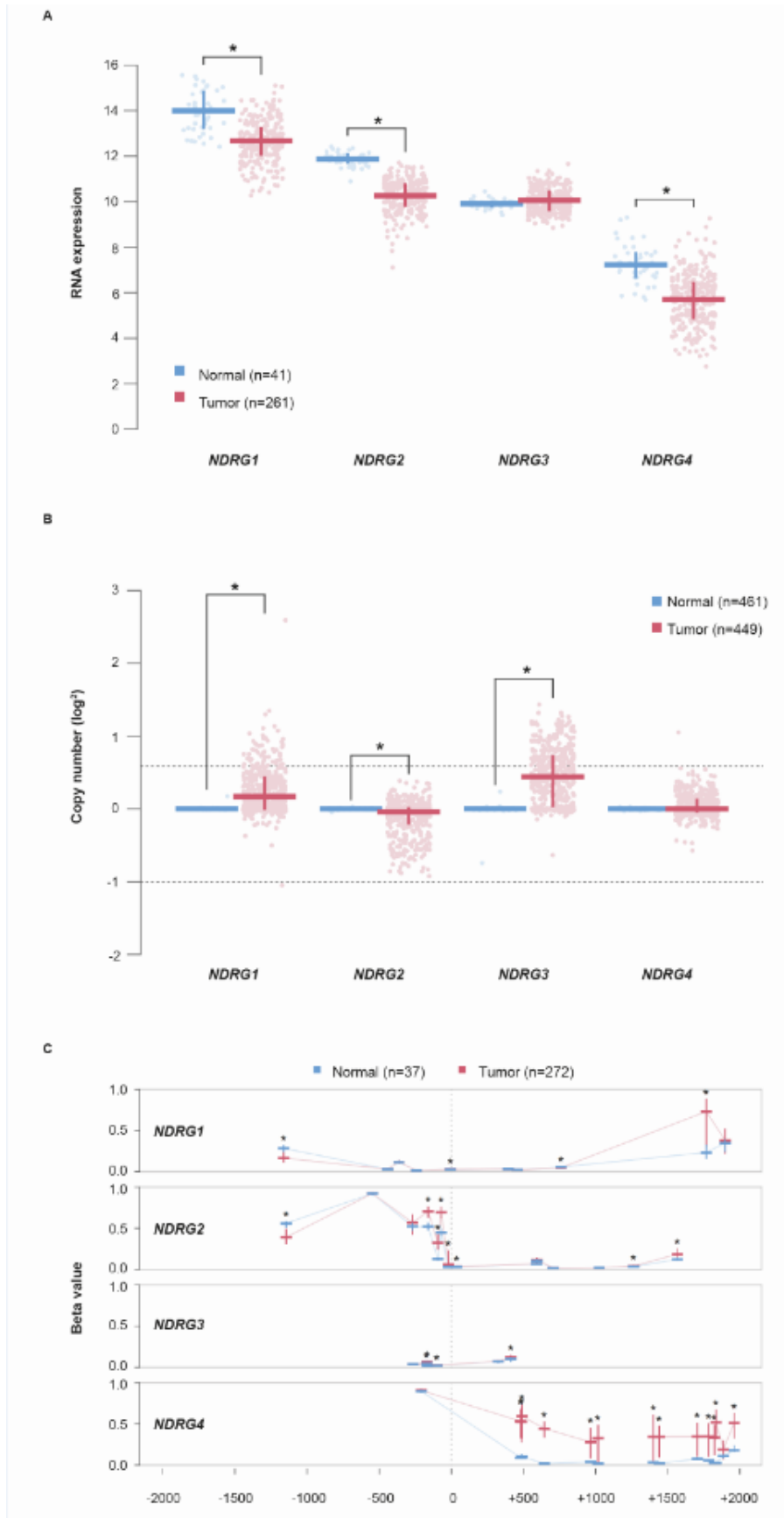


Figure 2. Differences in *NDRGs* between normal and tumor tissue from CRC patients as shown by TCGA data. **A.** TCGA-derived RNA expression analysis reveals, in agreement with the literature, the reduced expression of *NDRG1*, 2 and 4 in tumor tissue (n=261) compared to normal tissue (n=41). *NDRG3* expression on the other hand is very slightly, but not significantly increased. Statistical test; Wilcoxon rank sum test. * Indicate P<0.001 **B.** TCGA copy number data show that, compared to normal samples (n=461), tumor tissues (n=449) have a significantly increased copy number of *NDRG1* and *NDRG3*, a significantly reduced copy number of *NDRG2*, and an unaltered level of *NDRG4*. The dotted lines represent the copy number ratios with the upper one indicating an extra copy (3/2) and the lower representing loss of a copy (1/2). Statistical test; Wilcoxon rank sum test. * Indicate P<0.001 **C.** The CpG methylation status of TCGA samples indicates that each *NDRG* is more frequently methylated in tumor tissues (n=272) compared to normal tissues (n=37). In contrast to the few CpGs that are methylated in the *NDRG1* (4/10) and *NDRG3* (4/7) gene, methylation occurs most frequently in the *NDRG4* (13/15) and *NDRG2* (8/14) gene in CRC tissues. Statistical test; Wilcoxon rank sum test. * Indicate P<0.001

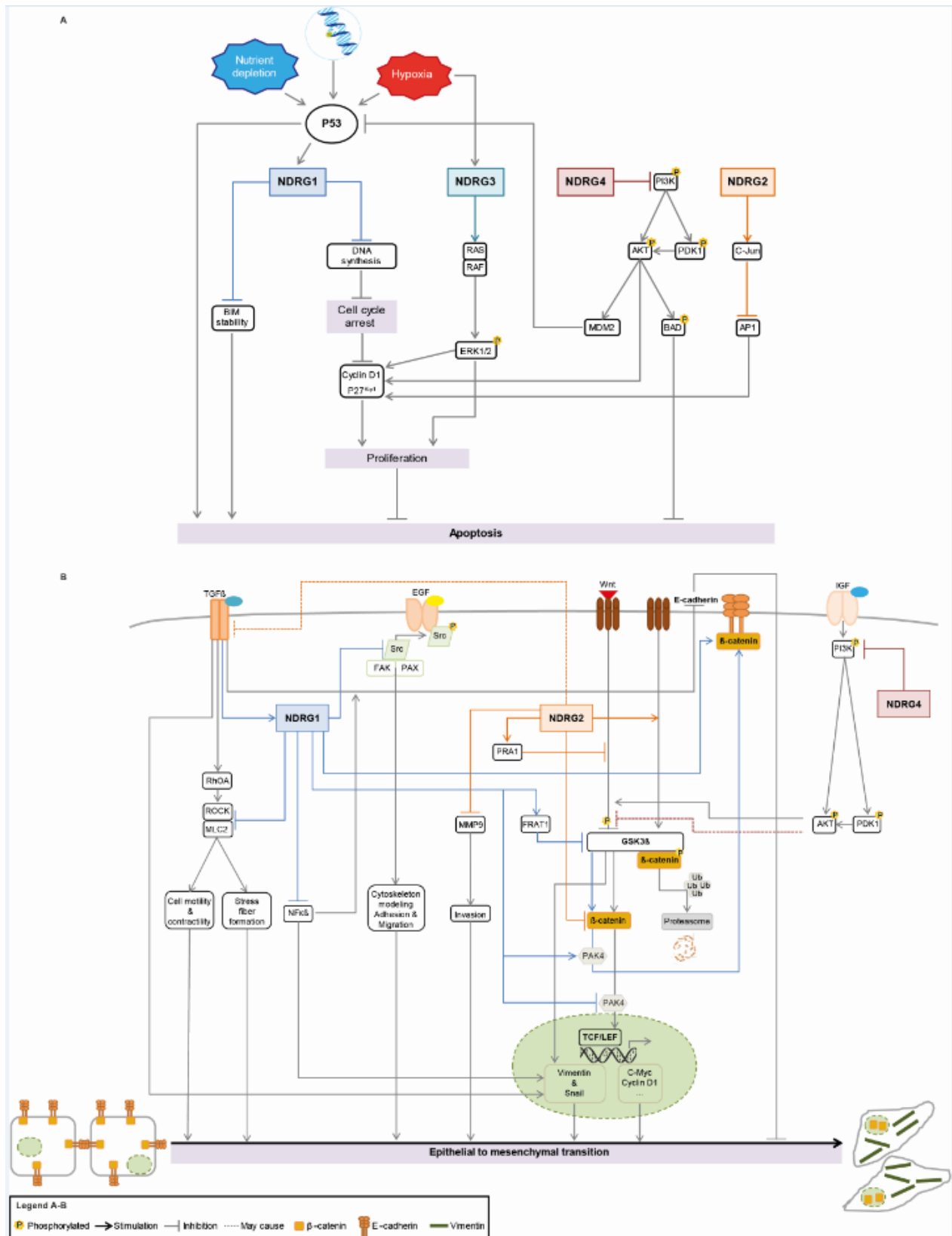


Figure 3. *Common pathways of NDRG1, 2, 3 and 4.* **A.** The impact of all four NDRG family members on the proliferative and apoptotic pathway during colorectal tumorigenesis. While each NDRG is able to regulate different pathways, all four members can also indirectly affect Cyclin D1 and P27^{Kip1}, thereby influencing the rate of proliferation and apoptosis. **B.** NDRG1, 2 and 4 are able to modulate the epithelial-mesenchymal transition (EMT) during colorectal carcinogenesis. The most commonly affected target by all three members is GSK3 β / β -catenin: i.e. they all prevent nuclear translocation of β -catenin, leading to inhibition of the EMT. In addition, each individual member affects different pathways to suppress the EMT. Grey lines indicate normal pathways (other critical pathways are omitted for clarity); colored lines represent the influence by each NDRG family member and dotted lines represent possible regulatory paths. **Abbreviations in A-B:** ERK1/2, Extracellular signal-regulated kinases; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, Protein kinase B; PDK1, Pyruvate Dehydrogenase Kinase 1; MDM2, E3 ubiquitin-protein ligase; BAD, Bcl-2-associated death promoter; AP1, Activator protein 1; Cav-1, Caveolin-1; TGF β , Transforming growth factor beta; EGF, Epidermal growth factor; IGF, Insulin-like growth factor; Src, Proto-oncogene tyrosine-protein kinase; FAK, Focal adhesion kinase; PAX, Paired box; PRA1, Prenylated Rab acceptor protein 1; RhoA, Ras homolog gene family, member A; ROCK, Rho-associated protein kinase; MLC2, Myosin Light Chain 2; MMP9, Matrix metalloproteinase 9; FRAT1, Frequently rearranged in advanced T-cell lymphoma 1; PAK4, p21-activated kinase 4 ; NF κ β , nuclear factor kappa-light-chain-enhancer of activated B cells; TFC/LEF, T-cell factor/Lymphoid enhancer factor.

Funding

This work was supported by the KWF Kankerbestrijding grant (UM 2013-6075) obtained by Dr. Veerle Melotte. The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The human embryonic and fetal material was provided by the Joint MRC/Wellcome Trust grant# 099175/Z/12/Z Human Developmental Biology Resource (<http://hdbr.org>).

Availability of data and materials

The results shown in the section: “Differential expression levels during intestinal carcinogenesis” are in part based upon data generated by the TCGA Research Network: <http://cancergenome.nih.gov/>. The data used for the analyses described in the section: “Expression pattern of the NDRGs in the developing and adult gut” were obtained from: [<https://gtexportal.org/home/>] the GTEx Portal on 06/19/2017. The human embryonic and fetal material was provided by the Joint MRC/Wellcome Trust grant# 099175/Z/12/Z Human Developmental Biology Resource (<http://hdbr.org>).

Author contributions

NV and SS performed the literature search; NV and VM designed the study; EB and AK analyzed the data derived from databases (EB, GTEx and mouse embryonic data; AK, TCGA data); CJM and NT assembled all data of the GTEx project; NV, EB, AK, RMWH, MvE and VM contributed

towards data interpretation; NV, SS, EB and AK generated the figures; NV, SS and VM wrote the paper; EB, AK, CJM, NT, RMWH and MvE provided critical revisions of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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