EMBO reports

Loss of enteric neuronal Ndrg4 promotes colorectal cancer via increased release of Nid1 and Fbln2

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Dear Dr. Melotte,

Thank you for transferring your manuscript to EMBO reports. I now went through your manuscript, the referee reports from The EMBO Journal (attached below), and your revision plan. The referees acknowledge that the findings are of interest, but have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn.

Given the constructive referee comments, we would like to invite you to revise your manuscript as you suggested in your revision plan, with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a rebuttal letter. EMBO reports emphasizes novel functional over detailed mechanistic insight. Thus, we will not require addressing points regarding more refined mechanistic details experimentally. But we ask for strong in vivo relevance of the findings, and clear experimental support of the major conclusions.

Acceptance of your manuscript will depend on a positive outcome of a second round of review (using the same set of referees). It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

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Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Achim

Achim Breiling
Editor
EMBO reports

Referee #1:

The manuscript by Vaes et al., investigates the role of enteric Ndrg4 suppression in colorectal cancer progression. Interestingly, they discover that although intestinal adenoma does not occur with higher frequency, their size and level of aggressiveness are greatly enhanced in two cancer mouse models when Ndrg4 is removed. Proteomic analysis further identified two extracellular matrix molecules (Nid1 and Fbln2) with enhanced expression within the secretome of Ndrg4 mutant tissue. The overall intact structure/function of the gut, as well as the direct effect on Ndrg4-mutant cell secretome on intestinal organoid growth, suggest that the altered expression of secreted molecules from the ENS form the mechanism behind the acceleration in colorectal cancer. The authors can thus explain the role of Ndrg4, that previously only served as a methylated biomarker, and thus pave the way for new molecular targets to treat colorectal cancer. The manuscript represent a great advancement in the field. It is written in a succinct manner, is easy to follow and in most cases with sufficient detail to support major claims and conclusions. However, it will be important to solidify claims of the overall lack of phenotype in the structure/function of the ENS in Ndrg4 mutant mice, and discuss broader explanations to their findings given the extensive extrinsic innervation of the gut mucosa. The mixed nature of "ENS cultures" used, also call out for a more detailed description of those and further validation of which cells display the altered secretome phenotype.

Major Issues:
1) The authors conclude that the structure of the ENS in not affected in the absence of Ndrg4 expression. However, evidence for this claim is only shown in the myenteric plexus, which do not contain all ENS cell bodies, and only a minority of its projections. The ENS also constitutes of the submucosa which send dense processes to the epithelial layer (along with the sensory subset of the myenteric IPANs). Therefore, the submucosal plexus (number of HuC/D expressing cells), and villi innervation (Tuj1+ density or other suitable measure) should also be investigated. Especially since the submucosal ENS is more likely to be involved in the studied phenomena given its proximity and direct connections to the villi.

2) According to MouseBrain.org, Ndrg2 is also expressed in the remaining peripheral nervous system (DRG and sympathetic ganglia). It is therefore possible that the effects on tumour size seen in Ndrg4-/-APCmin/+ mutant mice are partly leverage from extrinsic fibers innervating the mucosa. Most likely, the ENS plays the most important role (as shown in vitro), but it is important to speculate in the discussion on the possible role of the extensive extrinsic innervation for the phenotypes described and for colorectal cancer.

3) Figure 4B: How was data in Figure 4B derived? Did you perform any computational analysis? The gene-search function in mousebrain.org does not give this output representations. Does the ENS correspond to both neurons and glia in your picture? Please provide information on how the analysis and representation was made.

4) Given the importance of the ENS culture secretome for the conclusion of the study, it is important to present details of the cellular constitution of these cultures. From the methodological sparse description it appears as if whole muscle peels are digested, indicating that the resulting cultures would contain mainly muscle cells and only a minority of enteric neurons. Please describe the cell composition of the cultures - which percentage of ENS cells do they contain? Does the impurity of these cultures call out for alternative interpretation of which cells produce the secretome? Please provide evidence for that the enhanced Nid1/Fbln2 expression occurs specifically in ENS cells and not throughout the cultures.

Minor Issues:

1) Supplementary Material and Methods: The description of RNA isolation and qRT-PCR is not detailed enough. Please provide the full details of primary ENS cultures (where are they isolated from, how long were they cultured for). You may also refer back to description in Material and Methods if appropriate.

2) Figure 4 legend. C,E indicates "expressed within the myenteric plexus and by cells of the ENS". Please correct the legend.

Referee #2:

In this article, Vaes et al investigate the role of Ndrg4 in colorectal cancer development by using mouse models and in vitro culture system. They first analyzed the phenotype in Ndrg4 knockout mice in normal state, and confirmed that intestinal epithelium and enteric neurons are not significantly altered compared to control mice. Then they challenged tumorigenic analysis by using AOM administration and ApcMin mice, and demonstrated that knockout of Ndrg4 increases the size of intestinal adenoma. Transcriptomic analysis of cultured ENS suggested that genes related to vesicle formation/transportation are downregulated in Ndrg4-/- neuronal cells, and when co-cultured with intestinal organoids, intestinal organoids grew faster with Ndrg4-/- ENS than with Ndrg4+/+ ENS. Proteomics analysis revealed that Ndrg4-/- ENS secreted higher levels of Nidogen1 and Fibulin2, extracellular matrix proteins, which promoted migration of human colorectal cancer cell lines in vitro.

The manuscript is well written and the authors have done detailed quantification and various assays. In vitro culture systems and proteomics results are intriguing and very solid. However, phenotypic differences in mouse tumor models are not striking, and the mechanism where Nidogen1 and Fibulin2 promote colon cancer development remains unclear, and does not fully explain the findings in mice. Overall, while efforts made by the authors are quite appreciated, the significance and impact of this study are somewhat limited. Other detailed comments are listed below.

1. Introduction. They describe the background as to nerve-cancer interaction, which is good, but there is no detailed description about why they got interest in the specific molecule Ndrg4. While the authors seem to have published a few studies focusing on Ndrg4 in the past, it would be appreciated if they describe more detailed information about Ndrg4, such as biological function, role in human diseases, relationship to colon cancer, etc.

2. In Figure1, the authors compared the numbers of differentiated cell types and proliferating cells in the intestine and colon. However, while Paneth cells can be found normally only in the small intestine, the other cell types they analyzed exist both in the small intestine and colon. The authors should show the images/quantification from both organs. Also, it would be helpful if the authors compare the stem cell marker expression, such as Lgr5 or Olfm4, given that later the authors showed that Ndrg4-/- ENS promotes intestinal organoid growth, which largely depend on stem cell function.

3. They crossed Ndrg4F/F mice with Villin-Cre mice, and found no significant difference. However, since Ndrg4 appears to be expressed in neuronal cells, what is really needed here is to cross with neuron-specific Cre transgenic lines, and induce tumors in the intestine/colon.

4. How nidogen1 and fibulin2 promote colon cancer development is largely unclear. Fig.4C suggests

that both nidogen1 and fibulin2 are expressed in colonic myenteric plexus - how do they reach to epithelium or tumor cells? Are there any expression within adenoma? How do these molecules increase cell migration - can nidogen1 and fibulin2 directly bind to cancer cells and stimulate certain signaling pathways in cancer cells? These points are not addressed. Also, in vitro study suggested that nidogen and fibulin promote migration but not proliferation. However, in Ndrg4-/- mice, significant difference was found only in tumor size, which are mainly affected by cell proliferation. More explanation is needed for linking the in vitro and in vivo findings.

Referee #3:

In this study, Vaes et al. describe a role for Ndrg4, an ENS-specific CRC biomarker, in regulating intestinal tumorigenesis. Loss of Ndrg4 in healthy mice had no significant effect on overall intestinal morphology and physiology, but two independent Ndrg4-/- CRC mouse models displayed increased adenoma loads relative to Ndrg4+/+ CRC mice. Moreover, Ndrg4-/- ENS cultures showed downregulation in the expression of several genes involved in vesicle transport and exocytosis, and intestinal epithelial organoids exposed to Ndrg4-/- ENS culture media grew more rapidly, pointing towards a role for Ndrg4 in regulating the secretion of factors that promote organoid growth. Finally, the authors identified NID1 and FBLN2 as two ECM proteins significantly upregulated in the Ndrg4-/- ENS culture secretome, which, when introduced into CRC cell culture media, were sufficient to accelerate cell migration rates. Given that NID1 and FBLN2 are also upregulated in human CRC secretomes, the findings on Ndrg4 in this study could potentially have therapeutic translational outcomes for the treatment of human CRC.

Overall, this is a thorough study of the role of Ndrg4 using a number of in vivo and in vitro CRC models. It also puts forth an interesting perspective on the role of the nervous system as part of the tumour microenvironment in driving CRC progression. However, there are several major areas for improvement that should be worked on to better support the findings in this manuscript (detailed below). In general, some conclusions made were either inaccurate (due to p-values that were insignificant), or were not well substantiated by the experimental results. Another source of concern is the interpretation of results across a diverse range of models (CRC mouse models, ENS and intestinal organoid co-cultures, and CRC cell lines). For example, identification of NID1 and FBLN2 from ENS-intestinal organoid co-cultures was then incorporated in the analysis of cell proliferation and migration in CRC cell lines without a careful evaluation of whether the results are transferrable across systems. Moreover, given that in vivo and in vitro systems display important physiological differences, the authors could better support their in vitro work by confirming more of their findings with their CRC mouse models, for example by checking for NID1 and FBLN2 within Ndrg4-/- CRC mice. Finally, while a detailed mechanism may be out of the scope of this paper, the authors could better clarify how the loss of Ndrg4 promotes the secretion of NID1 and FBLN2, as it appears counter-intuitive that loss of Ndrg4 increases secretion but also results in the downregulation of several genes involved in vesicle transport/exocytosis.

Specific major points:
1. A major point of concern throughout the manuscript is the fact that many conclusions were drawn from results that are not considered statistically significant, based on the authors' own p-value cut-off of 0.05 (indicated in their Methods section). This include:
a. In the section corresponding to Figure 2, the authors concluded that adenomas from Ndrg4-/- mice displayed higher mean nuclear b-catenin immunoreactivity than those from Ndrg4+/+ mice, despite a p-value of 0.06 and 0.085 for the APCMin/+ model and the AOM-induced model

respectively.
b. In Figure 3D, a number of the genes identified to be downregulated as a result of Ndrg4 knockdown in ENS cultures should be excluded from the list, as they have p-values higher than 0.05: Nsf ($p = 1.000$), Vamp2 ($p = 0.151$), Snap25 ($p = 0.690$), Stx1a ($p = 0.222$), Rab3a ($p = 0.056$), Cadps ($p = 0.222$). As a result, the authors' conclusion needs to be modified to account for the result showing that none of the genes associated with vesicle docking and fusion was affected by Ndrg4 downregulation in ENS cultures.
c. In Figure 5C, the conclusion that treating HCT116 cells with NID1 and FBLN2 improved proliferation after 96 hours should be revised, as the p-value of 0.094 suggests that the difference in proliferation is not significant. There are similar issues for Figure 5D, with $p = 0.087$ for addition of FBLN2 after 24 hours, and $p = 0.093$ for addition of NID1 and FBLN2 after 48 hours, and for Figure 5E, $p = 0.067$ for addition of FBLN2 after 48 hours.

2. For Figure 4, the authors identified NID1 and FBLN2 as two ECM proteins that are significantly enriched in the secretome of Ndrg4-/- ENS cell cultures (compared to Ndrg4+/+), and found that NID1 and FBLN2 are also highly expressed in the ENS through immunostainings of wildtype tissue sections and Ndrg4+/+ ENS cultures. However, it would also be important to assess NID1 and FBLN2 expression (both at the RNA and protein levels) within Ndrg4-/- tissue sections / ENS cultures, and compare their expression to the levels found in the corresponding Ndrg4+/+ cells.

3. In the section of the paper corresponding to Figure 5, the authors utilized two CRC cell lines (HCT116 and Caco-2) to assess the effect of adding NID1 and/or FBLN2 on their proliferation and migration capabilities. However, the authors identified NID1 and FBLN2 from secretomes of ENS cultures that they showed to have an impact on intestinal epithelial organoid growth rates (Figures 3 and 4). Thus, the authors could also consider testing the effect of NID1 and/or FBLN2 administration on intestinal epithelial organoid growth (in addition to the CRC cell lines), as this will better support their conclusion that these specific factors derived from the ENS secreted fraction can regulate intestinal cell growth.

4. Given the clinical translatability of the findings from this paper, the authors could better support their results by performing rescue experiments to reintroduce Ndrg4 to Ndrg4-depleted cells and measure effects on cell proliferation and migration.

5. In the discussion section, the authors suggest that Ndrg4 knockdown promotes epithelial-mesenchymal transition of intestinal epithelial cells. However, their current set of findings do not directly support this conclusion, as they have only conclusively shown that the Ndrg4-/- cancer mouse models display larger adenomas, and in fact do not display any differences in tumour incidence (initiation). Thus, the authors should revise this statement, or better support their statement by checking for markers of EMT.
Minor points:
1. In the section of the paper corresponding to Figure 2, the authors describe differences in mean nuclear b-catenin immunoreactivity between adenomas from Ndrg4+/+ and Ndrg4-/- CRC mouse models by comparing some measured values listed in the text. However, it is unclear based on these values how many adenomas were evaluated for each condition, the spread of the data, or how these numbers were obtained (for example, were they normalized to a reference value?). The authors could improve on this result by presenting their data in a graph provided in Figure 2, and support this data with accompanying immunostaining images to better visualize the nuclear localization of b-catenin in these samples.

2. In Figure 3D, the authors present their RNA expression analysis for a number of genes that were

downregulated as a result of Ndrg4 knockdown in ENS cultures. These hits could be further validated by protein expression assays such as western blots or immunostainings. Moreover, given that the identified hits suggest a role for Ndrg4 in vesicle transport, an immunostaining analysis could also reveal changes in subcellular localization, apart from changes in overall expression levels.

3. The reference to Figure 5C in the sentence "As shown in Fig. 5C, the singular addition of either NID1 or FBLN2 did not affect the proliferation rate of HCT116 cells..." was wrongly indicated, the corresponding figure for this result should be Figure 5B.

Referee #1:

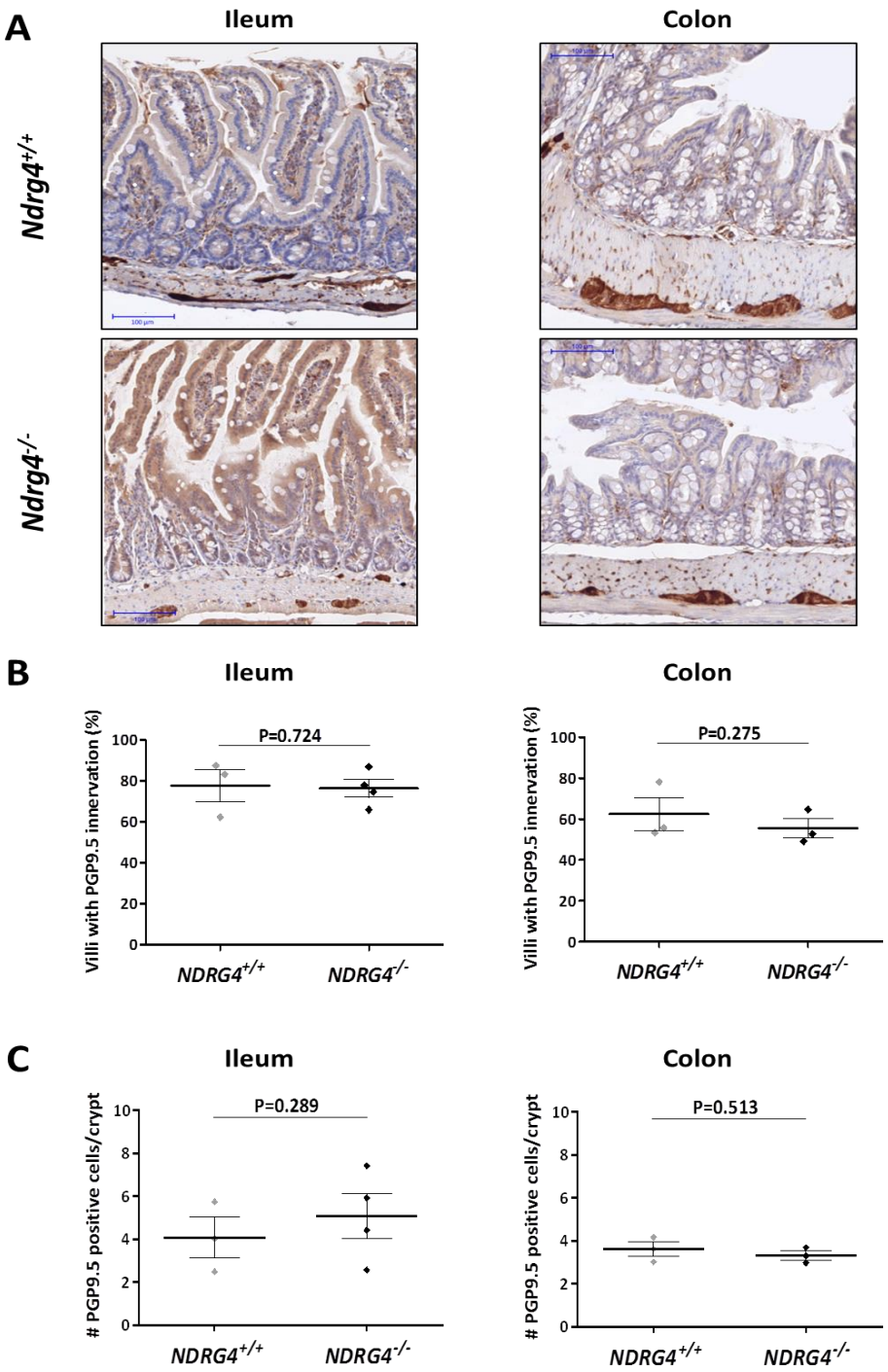
The manuscript by Vaes et al., investigates the role of enteric Ndrg4 suppression in colorectal cancer progression. Interestingly, they discover that although intestinal adenoma does not occur with higher frequency, their size and level of aggressiveness are greatly enhanced in two cancer mouse models when Ndrg4 is removed. Proteomic analysis further identified two extracellular matrix molecules (Nid1 and Fbln2) with enhanced expression within the secretome of Ndrg4 mutant tissue. The overall intact structure/function of the gut, as well as the direct effect on Ndrg4-mutant cell secretome on intestinal organoid growth, suggest that the altered expression of secreted molecules from the ENS form the mechanism behind the acceleration in colorectal cancer. The authors can thus explain the role of Ndrg4, that previously only served as a methylated biomarker, and thus pave the way for new molecular targets to treat colorectal cancer. The manuscript represent a great advancement in the field. It is written in a succinct manner, is easy to follow and in most cases with sufficient detail to support major claims and conclusions. However, it will be important to solidify claims of the overall lack of phenotype in the structure/function of the ENS in Ndrg4 mutant mice, and discuss broader explanations to their findings given the extensive extrinsic innervation of the gut mucosa. The mixed nature of "ENS cultures" used, also call out for a more detailed description of those and further validation of which cells display the altered secretome phenotype.

Major Issues:

1) The authors conclude that the structure of the ENS in not affected in the absence of Ndrg4 expression. However, evidence for this claim is only shown in the myenteric plexus, which do not contain all ENS cell bodies, and only a minority of its projections. The ENS also constitutes of the submucosa which send dense processes to the epithelial layer (along with the sensory subset of the myenteric IPANs). Therefore, the submucosal plexus (number of HuC/D expressing cells), and villi innervation (Tuj1+ density or other suitable measure) should also be investigated. Especially since the submucosal ENS is more likely to be involved in the studied phenomena given its proximity and direct connections to the villi.

Unfortunately, we were not able to investigate the innervation within the submucosal plexus as this required the setup of a new mouse breeding to isolate the submucosal plexus from *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice.

Nevertheless, we performed immunohistochemistry on paraffin-embedded small and large intestinal Swiss rolls of our *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice (n=3 per genotype) using the neuronal marker PGP9.5 (Z5116, Dako). As depicted in the representative images below and their concomitant quantification, deletion of *Ndrg4* has no influence on the innervation of the villi in the small and large intestine (**Revision fig. 1**). These data have been added within the results section on page 6: *"Finally, also anti-PGP9.5 immunohistochemistry did not reveal any differences in the mucosal innervation of the small and large intestine of Ndrg4*$^{-/-}$ *and Ndrg4*$^{+/+}$ *mice (data not shown)."*.



Revision figure 1. Deletion of *Ndrg4* has no influence on mucosal innervation. (**A**) Representative images of intestinal sections of *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice stained with anti-PGP9.5 (scale bar, 100 µm). (**B-C**) Quantification of the percentage of villi exposing anti-PGP9.5 reactivity (**B**) and of the number of PGP9.5 positive cells/fibers per villus or crypt (**C**) shows a similar mucosal innervation in *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice (n=3 per genotype). Data are represented as mean ± SEM and p-values determined with the Mann-Whitney U test (IBM SPSS statistics 25).

2) According to MouseBrain.org, Ndrg2 is also expressed in the remaining peripheral nervous system (DRG and sympathetic ganglia). It is therefore possible that the effects on tumor size seen in Ndrg4-/-

APCmin/+ mutant mice are partly leverage from extrinsic fibers innervating the mucosa. Most likely, the ENS plays the most important role (as shown in vitro), but it is important to speculate in the discussion on the possible role of the extensive extrinsic innervation for the phenotypes described and for colorectal cancer.

In the original manuscript we did not speculate about the contribution of extrinsic innervation to the tumor development. We therefore added this interesting suggestion in the discussion (page 10): *"Importantly, we have to keep in mind that within this constitutive Ndrg4 knockout model, not only enteric neurons, but also peripheral nerves are deficient for Ndrg4. Given that extrinsic denervation can reduce carcinogenesis, as seen in other cancer types (6,9,12), it may be speculated that Ndrg4 deficiency in peripheral nerves could contribute to the observed phenotype.".*

3) Figure 4B: How was data in Figure 4B derived? Did you perform any computational analysis? The gene-search function in mousebrain.org does not give this output representations. Does the ENS correspond to both neurons and glia in your picture? Please provide information on how the analysis and representation was made.

The data shown in Fig. 4B within the manuscript are retrieved from the Linnarsson's online dataset (Zeisel A *et al*, 2018; http://linnarssonlab.org/data/) and are reflected as such. We have not performed any additional computational analyses. The color scale in our manuscript deviates from the one depicted by the Linnarsson's lab because the color scale in the online dataset differs per gene and the highest expression is reflected in the darkest color with all other expression values compared to this value. Since Nid1 and Fbln2 are shown together in one figure within our manuscript, we made use of one color scale suitable for the visualization of both genes. We also added the reference from *Zeisel A et al, 2018* within the manuscript text on page 8: *"We examined the gene expression of Nid1 and Fbln2 in the nervous system using the Mouse Brain atlas from the Linnarsson Lab (33,**34**).".*

The "ENS" within this figure indeed corresponds to both enteric neurons and glial cells, as it reflects "enteric nervous system". Consequently, we added *"(i.e. enteric neurons and glial cells)"* following *the "ENS, enteric nervous system"* abbreviation in the legend of Fig. 4B on page 25.

4) Given the importance of the ENS culture secretome for the conclusion of the study, it is important to present details of the cellular constitution of these cultures. From the methodological sparse description it appears as if whole muscle peels are digested, indicating that the resulting cultures would contain mainly muscle cells and only a minority of enteric neurons. Please describe the cell composition of the cultures - which percentage of ENS cells do they contain? Does the impurity of these cultures call out for alternative interpretation of which cells produce the secretome? Please provide evidence for that the enhanced Nid1/Fbln2 expression occurs specifically in ENS cells and not throughout the cultures.

The reviewer is indeed correct that these cultures are generated by digesting the LMMP (longitudinal muscle and myenteric plexus) and also contained cell types other than neurons and glial cells. Although we have not assessed the exact composition of/or percentages of cells within our cultures, we have applied a commonly used protocol to isolate and culture ENS cells (Lowette K *et al*, 2014; Herdewyn S *et al*, 2014). As we are aware that these cultures also contain non-neuronal cells e.g. muscle cells and fibroblasts (as visible in Fig. 3C of the manuscript) we cultured these cells for a maximum of seven days to prevent them from overgrowing enteric neurons and glia. As we showed that Ndrg4 expression is restricted to enteric neurons (Vaes *et al*, NGM, 2017), we can conclude that this enteric neuronal deficiency of *Ndrg4* is the driving force for the differential presence of proteins within the ENS cell secretome.

To clarify the mixed composition of the cultures, we have adapted the description of the "primary murine (3D)- cultures" in the methods section by adding the following sentences on page 15: *"The cells **(mixture containing neurons, glial cells and e.g. smooth muscle cells and fibroblasts)** were cultured in a 12-well plate at 37°C (95% O_2/5% CO_2)."* and *"The medium was changed every two days and cells **were cultured for a maximum of seven days in order to prevent overgrowth by non-ENS cell types. At that point, cells were harvested (pool three wells of a 12-well plate) for further analysis."**.*

Minor Issues:

1) Supplementary Material and Methods: The description of RNA isolation and qRT-PCR is not detailed enough. Please provide the full details of primary ENS cultures (where are they isolated from, how long were they cultured for). You may also refer back to description in Material and Methods if appropriate.

We have adapted the Supplementary Materials and Methods section: *"Total RNA was isolated from primary mouse ENS cells **(derived and cultured as described in the primary murine (3D)-culture paragraph of the Materials and Methods section)** using TRIzol® reagent and the purelink RNA mini Kit (Ambion, Life Technologies) according to manufacturer's instructions."* .

2) Figure 4 legend. C,E indicates "expressed within the myenteric plexus and by cells of the ENS". Please correct the legend.

We have adapted the figure legend on page 25 accordingly: *"(**C-E**) Representative immunohistochemistry (**C**) and more detailed immunofluorescence (**D, E**) labeling indicate that Nid1 (**C, D**) and Fbln2 (**C, E**) are expressed within the myenteric plexus and **by primary ENS-cells**."*.

Referee #2:

In this article, Vaes et al investigate the role of Ndrg4 in colorectal cancer development by using mouse models and in vitro culture system. They first analyzed the phenotype in Ndrg4 knockout mice in normal state, and confirmed that intestinal epithelium and enteric neurons are not significantly altered compared to control mice. Then they challenged tumorigenic analysis by using AOM administration and ApcMin mice, and demonstrated that knockout of Ndrg4 increases the size of intestinal adenoma. Transcriptomic analysis of cultured ENS suggested that genes related to vesicle formation/transportation are downregulated in Ndrg4-/- neuronal cells, and when co-cultured with intestinal organoids, intestinal organoids grew faster with Ndrg4-/- ENS than with Ndrg4+/+ ENS. Proteomics analysis revealed that Ndrg4-/- ENS secreted higher levels of Nidogen1 and Fibulin2, extracellular matrix proteins, which promoted migration of human colorectal cancer cell lines in vitro.
The manuscript is well written and the authors have done detailed quantification and various assays. In vitro culture systems and proteomics results are intriguing and very solid. However, phenotypic differences in mouse tumor models are not striking, and the mechanism where Nidogen1 and Fibulin2 promote colon cancer development remains unclear, and does not fully explain the findings in mice. Overall, while efforts made by the authors are quite appreciated, the significance and impact of this study are somewhat limited. Other detailed comments are listed below.

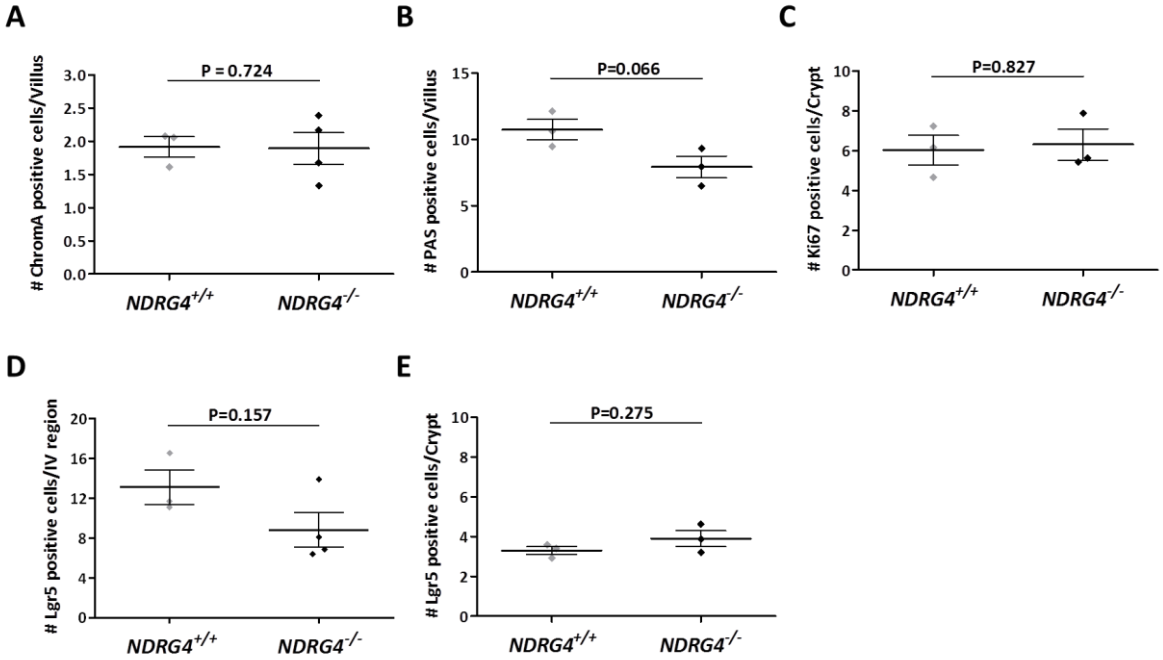
1. Introduction. They describe the background as to nerve-cancer interaction, which is good, but there is no detailed description about why they got interest in the specific molecule Ndrg4. While the authors seem to have published a few studies focusing on Ndrg4 in the past, it would be appreciated if they describe more detailed information about Ndrg4, such as biological function, role in human diseases, relationship to colon cancer, etc.

> We have adapted the final paragraph of the introduction (page 4) in an attempt to include more information regarding Ndrg4: *"Previously, we revealed that the N-Myc Downstream-Regulated Gene 4 (NDRG4), one of the most accurate DNA methylation-based biomarkers for the early detection of CRC (20,21), is specifically expressed in the ENS (22,23). Even though NDRG4 is an established DNA-methylation based biomarker (20,21), knowledge regarding its functional importance is sparse, yet seems to affect key developmental and carcinogenic processes, including cellular proliferation and differentiation (23-25). Moreover, NDRG4 has been shown to be involved in vesicle trafficking and thus possibly secretory actions (26,27). Given the extensive crosstalk between the ENS and the intestinal epithelium, we here investigate if enteric neuronal NDRG4 influences the intestinal (tumour) epithelium."*.

2. In Figure1, the authors compared the numbers of differentiated cell types and proliferating cells in the intestine and colon. However, while Paneth cells can be found normally only in the small intestine, the other cell types they analyzed exist both in the small intestine and colon. The authors should show the images/quantification from both organs. Also, it would be helpful if the authors compare the stem cell marker expression, such as Lgr5 or Olfm4, given that later the authors showed that Ndrg4-/- ENS promotes intestinal organoid growth, which largely depend on stem cell function.

As suggested by the reviewer, we have quantified the intestinal cell types within the small intestine. Similar as in the colon (Fig. 1A within manuscript), these cell populations were evenly distributed and did not significantly differ between the *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice (**Revision fig. 2A-C below**). In addition, we also evaluated the number of Lgr5 positive cells (Ab71225, Abcam) in both the ileum and colon of *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice. This analysis showed that this population is evenly present in small and large intestinal sections of *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice (**Revision fig. 2D-E below**), indicating that the stem cell population is not altered within our *Ndrg4*$^{-/-}$ model.

Consequently, we have embedded these results within the manuscript and Fig. 1A and adapted the paragraph on page 6: *"In-depth histological analyses of intestinal segments of Ndrg4*$^{-/-}$ *and Ndrg4*$^{+/+}$ *mice revealed a normal intestinal epithelial architecture: i.e. uniform presence of alkaline phosphatase in the enterocyte brush border and similar number and distribution of Paneth (Lysozyme; p=0.537, small intestine) and of neuroendocrine (Chromogranin A; p=0.304), Goblet (PAS; p=0.958),* ***stem (Lgr5; p=0.275)*** *and proliferating cells (Ki67; p=0.543****) in the colon*** *(****Fig. 1A****). **Similar numbers and distributions of neuroendocrine-, goblet-, stem- and proliferating cells were observed within the small intestine (data not shown).**"*, as well as the figure legend of Fig. 1. on page 23: *(**A**) Representative microscopic views of Ndrg4*$^{+/+}$ *and Ndrg4*$^{-/-}$ *murine intestinal sections (n=4, 12 months of age) reveal an even distribution of alkaline phosphatase along the enterocyte brush border, and a similar number and distribution of Paneth cells (Lysozyme) in the small intestine; and neuroendocrine (Chromogranin A), Goblet (PAS+),* ***stem (Lgr5+)*** *and proliferating (Ki67) cells in the colon."*.



Revision figure 2. Loss of *Ndrg4* does not affect intestinal cell populations. (**A-C**) The quantification of intestinal cells within the ileum reveals a similar distribution and number of neuroendocrine cells (Chrom A, **A**), Goblet (PAS+, **B**) and proliferating (Ki67, **C**) cells in *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice. (**D-E**) Quantification of the Lgr5 positive cell population within the intervillus region (IV region) in the small intestine (**D**) and colon (**E**). Data are represented as mean ± SEM and p-values calculated using the Mann-Whitney U test (IBM SPSS statistics 25).

3. They crossed Ndrg4F/F mice with Villin-Cre mice, and found no significant difference. However, since Ndrg4 appears to be expressed in neuronal cells, what is really needed here is to cross with neuron-specific Cre transgenic lines, and induce tumors in the intestine/colon.

The reviewer is indeed correct that a neuron-specific Cre-transgenic line would be a more suitable model. However, at the time of our experiments we had no access to an enteric neuron-specific Cre driver line.

4. How nidogen1 and fibulin2 promote colon cancer development is largely unclear. Fig.4C suggests that both nidogen1 and fibulin2 are expressed in colonic myenteric plexus - how do they reach to epithelium or tumor cells? Are there any expression within adenoma? How do these molecules increase cell migration - can nidogen1 and fibulin2 directly bind to cancer cells and stimulate certain signaling pathways in cancer cells? These points are not addressed. Also, in vitro study suggested that nidogen and fibulin promote migration but not proliferation. However, in Ndrg4-/- mice, significant difference was found only in tumor size, which are mainly affected by cell proliferation. More explanation is needed for linking the in vitro and in vivo findings.

Point 1: How nidogen1 and fibulin2 promote colon cancer development is largely unclear. Fig.4C suggests that both nidogen1 and fibulin2 are expressed in colonic myenteric plexus - how do they reach to epithelium or tumor cells? How do these molecules increase cell migration - can nidogen1 and fibulin2 directly bind to cancer cells and stimulate certain signaling pathways in cancer cells?

Future studies have are necessary to elucidate the exact mechanism by which Nid1 or Fbln2 reach the intestinal epithelium or tumor cells and promote carcinogenesis. However, it is possible that these molecules are transported within vesicles towards the intestinal epithelial layer. NID1 has already been associated with extracellular vesicles secreted from hepatic tumor cells (Xiaowen M. et al, 2020) and mostly with exosomes in the lung cancer secretome (Alečković M et al, 2017). In addition, we debated about their possible targets (on CRC cells) at the end of the paragraph in the discussion (page 11): *"Further studies are needed to identify which specific receptors are targeted by NID1 and FBLN2, e.g. β1-integrin receptors (44,46), and thereby mediate the observed effects in this study.".* Given that different subtypes of β-integrin receptors are abundantly present on CRC cells, NID1 and FBLN2 are likely to target and bind CRC cells. Consequently, we further adapted the manuscript (mid-page 11) accordingly: *"Similar as in other cancers (44,46,48), our assays showed that NID1/FBLN2 especially enhance the migration rate of epithelial-like HCT116 and Caco-2 cells,* ***most likely via binding and activation of (one of) the abundantly present β-integrin receptor family members (49,50).****"*.

Point 2: Are there any expression within adenoma?

To assess whether both proteins are differentially expressed within adenoma and cancerous epithelium, we used data available within the GSE77955 and GSE100179 (affymetrix 2.0 array; adenoma vs. normal) and TCGA dataset (CRC vs. normal). Using the GSE77955 dataset, we observed that the expression of NID1 and FBLN2 is (significantly) lower (p_{NID1}=0.00026 and p_{FBLN2}=0.54) in adenoma tissues (n=18) than in normal epithelium (n=13). Similarly, the GSE100179 dataset revealed a significantly lower expression of both NID1 and FBLN2 in adenoma versus normal colon tissue (n=20 samples each; p_{NID1}=0.00019 and p_{FBLN2}<0.0001). In addition, TCGA data

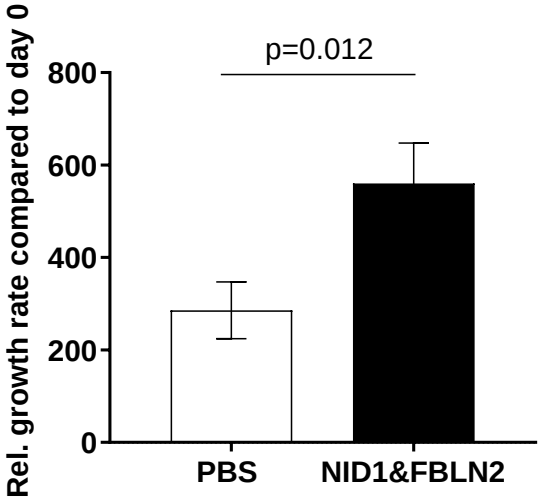
(n_{normal}=51 vs. n_{CRC}=383) revealed a (significantly) lower expression of both proteins in intestinal tumors compared to normal intestinal tissue sections (Wilcoxon rank sum test: p_{NID1}=0.083 and p_{FBLN2}<0.0001), even though significantly elevated levels of both proteins were found in the CRC secretome (**Fig. 5E** within the manuscript, p<0.0001 for both). Interestingly, expression data derived from the TCGA database showed that normal breast (n=114) and lung (n=59) tissues express (significantly) higher levels of NID1 and FBLN2 than breast (n=1104) and lung (n=517) tumor tissues (P_{FLBN2}<0.0001 and P_{NID1}<0.0001 and P_{FLBN2}=0.72 and P_{NID1}=0.034, respectively), even though the secretome of these cancer cell lines also harbor significantly up-regulated levels of NID1 and FBLN2 (Alečković M *et al,* 2017).

Consequently, we believe that tissue expression does not necessarily reflect the secreted fraction as such and future studies are necessary to investigate this concept.

Point 3: Also, in vitro study suggested that nidogen and fibulin promote migration but not proliferation. However, in Ndrg4-/- mice, significant difference was found only in tumor size, which are mainly affected by cell proliferation. More explanation is needed for linking the in vitro and in vivo findings.

As we aimed to translate our murine findings towards the human situation, we chose to administer these recombinant proteins to CRC cells. Even though we mainly observed an effect on cell migration, these data also showed that NID1/FBLN2 administration significantly enhanced the proliferation rate of HCT116 cells after 72 hours (p=0.044, Fig. 5C within the manuscript).

In addition, as also requested by reviewer 3, we have now explored the combinatorial effect of both recombinant human proteins (commercially available) on human intestinal organoids (HIOs) and found that the addition of NID1&FBLN2 within the matrigel dome (50µg/ml of both) significantly enhanced the relative growth rate of normal human intestinal organoids (**Revision fig. 3** below, p=0.012).



Revision figure 3. NID1&FBLN2 addition significantly enhances the proliferation rate of HIOs. Addition of NID1&FBLN2 to the Matrigel® dome significantly enhanced the relative growth rate after five days of culture as compared to the PBS control condition (i.e. similar ratio of PBS to matrigel as NID1&FBLN2 to matrigel). Data are represented as mean ± SEM with the p-value determined using the independent samples T-test (IBM SPSS statistics 25).

Consequently, we embedded these data within the results section, page 9: *"To explore whether the combinatorial treatment of NID1&FBLN2 affects the proliferation speed of the normal human intestinal epithelium, we exposed normal human intestinal organoids (HIOs) to NID1&FBLN2 within their Matrigel® dome and observed a significantly enhanced relative growth rate of HIOs (Fig. 5B, p=0.012)."*, and within Fig. 5. and its legend (page 25):*" (B) Addition of NID1&FBLN2 to the Matrigel® dome significantly enhances the relative growth rate of HIOs after five days of culture as compared to the PBS control condition. Data are represented as relative mean percentage ±SEM versus day 0, with the p-value determined using a two-tailed, unpaired t-test.*". Finally, we also included a further description of this experimental setup within the materials and methods section on page 16: '**Primary murine (3D-) cultures**': *"Primary human intestinal organoids (HIOs) were kindly provided by the group of Dr. T. Wolfs (METC 16-4-185). Similar as described for the murine IEOs, HIO were cultured in 1:1 human OGM/undiluted Matrigel® in the center of a 24-well plate, with refreshment of the medium every two days. Prior to experiments, the HIOs were disaggregated by gentle disruption of the Matrigel® dome with trypsin and incubation for ±2min at 37°C. After pelleting (400g, 5min, 4°C), the HIOs were resuspended in either one of the following conditioned Matrigel® domes: i.e. (1) 1:1 Matrigel®/Human OGM, (2) 1:1 Matrigel®/PBS or (3) 1:1 Matrigel®/NID1/FBLN2 (both at a final concentration of 50µg/ml) to assess the influence of NID1/FBLN2 on the growth rate of HIOs."* and within the '**Co-culture systems and live cell imaging paragraph'** (page 17): *"Similarly, HIO expansion was monitored daily for five consecutive days, starting immediately after solidification of the conditioned Matrigel® dome using the Leica DM3000 microscope (100× magnification). Relative growth rate was determined by measuring the circumference of the HIOs (ImageJ)."*.

Referee #3:

In this study, Vaes et al. describe a role for Ndrg4, an ENS-specific CRC biomarker, in regulating intestinal tumorigenesis. Loss of Ndrg4 in healthy mice had no significant effect on overall intestinal morphology and physiology, but two independent Ndrg4-/- CRC mouse models displayed increased adenoma loads relative to Ndrg4+/+ CRC mice. Moreover, Ndrg4-/- ENS cultures showed downregulation in the expression of several genes involved in vesicle transport and exocytosis, and intestinal epithelial organoids exposed to Ndrg4-/- ENS culture media grew more rapidly, pointing towards a role for Ndrg4 in regulating the secretion of factors that promote organoid growth. Finally, the authors identified NID1 and FBLN2 as two ECM proteins significantly upregulated in the Ndrg4-/- ENS culture secretome, which, when introduced into CRC cell culture media, were sufficient to accelerate cell migration rates. Given that NID1 and FBLN2 are also upregulated in human CRC secretomes, the findings on Ndrg4 in this study could potentially have therapeutic translational outcomes for the treatment of human CRC.
Overall, this is a thorough study of the role of Ndrg4 using a number of in vivo and in vitro CRC models. It also puts forth an interesting perspective on the role of the nervous system as part of the tumour microenvironment in driving CRC progression. However, there are several major areas for improvement that should be worked on to better support the findings in this manuscript (detailed below). In general, some conclusions made were either inaccurate (due to p-values that were insignificant), or were not well substantiated by the experimental results. Another source of concern is the interpretation of results across a diverse range of models (CRC mouse models, ENS and intestinal organoid co-cultures, and CRC cell lines). For example, identification of NID1 and FBLN2 from ENS-intestinal organoid co-cultures was then incorporated in the analysis of cell proliferation and migration in CRC cell lines without a careful evaluation of whether the results are transferrable across systems. Moreover, given that in vivo and in vitro systems display important physiological differences, the authors could better support their in vitro work by confirming more of their findings with their CRC mouse models, for example by checking for NID1 and FBLN2 within Ndrg4-/- CRC mice. Finally, while a detailed mechanism may be out of the scope of this paper, the authors could better clarify how the loss of Ndrg4 promotes the secretion of NID1 and FBLN2, as it appears counter-intuitive that loss of Ndrg4 increases secretion but also results in the downregulation of several genes involved in vesicle transport/exocytosis.

Specific major points:

1. A major point of concern throughout the manuscript is the fact that many conclusions were drawn from results that are not considered statistically significant, based on the authors' own p-value cut-off of 0.05 (indicated in their Methods section). This include:
a. In the section corresponding to Figure 2, the authors concluded that adenomas from Ndrg4-/- mice displayed higher mean nuclear β-catenin immunoreactivity than those from Ndrg4+/+ mice, despite a p-value of 0.06 and 0.085 for the APCMin/+ model and the AOM-induced model respectively.
b. In Figure 3D, a number of the genes identified to be downregulated as a result of Ndrg4 knockdown in ENS cultures should be excluded from the list, as they have p-values higher than 0.05: Nsf ($p = 1.000$), Vamp2 ($p = 0.151$), Snap25 ($p = 0.690$), Stx1a ($p = 0.222$), Rab3a ($p = 0.056$), Cadps ($p = 0.222$). As a result, the authors' conclusion needs to be modified to account for the result showing

that none of the genes associated with vesicle docking and fusion was affected by Ndrg4 downregulation in ENS cultures.
c. In Figure 5C, the conclusion that treating HCT116 cells with NID1 and FBLN2 improved proliferation after 96 hours should be revised, as the p-value of 0.094 suggests that the difference in proliferation is not significant. There are similar issues for Figure 5D, with p = 0.087 for addition of FBLN2 after 24 hours, and p = 0.093 for addition of NID1 and FBLN2 after 48 hours, and for Figure 5E, p = 0.067 for addition of FBLN2 after 48 hours.

To address the reviewers comment, we omitted the non-significant data from the manuscript (see below) and answered the reviewers concerns.

a.

As also discussed in Minor comment 1, we decided to not present the detailed data regarding the β-catenin staining in Fig. 2, because these data did not reach statistical significance. However, we felt that the obtained results were still of interest to our study and therefore choose to describe the data within the text. We now added *"non-statistically significantly"* within the sentence describing β-catenin reactivity on page 7.

b.

Based on the reviewer's comment we adapted the manuscript accordingly (page 7/8): ***"As shown in Fig. 3D, we tested the effect of Ndrg4 knockdown in primary murine ENS cell cultures on several genes involved in (i) vesicle formation Rabac1 and Nsf, (ii) vesicle docking and fusion: Vamp2, Snap25, Stx1a and Rab3a, and (iii) exocytotic release: Stxbp1, Caly and Cadps. A significantly reduced expression of Rabac1 (78.3%, p=0.016), Stxbp1 (18.3%, p=0.032), and Caly (82.1%, p=0.029) was observed in Ndrg4$^{-/-}$ ENS cultures."***.

In addition, we also adapted the concluding sentence regarding Ndrg4's involvement in vesicle trafficking (page 10): *"Consistent with the previously identified role for Ndrg4 in intracellular vesicle trafficking (26,27), we observed a* ***significantly reduced expression of Rabac1, Stxbp1 and Caly*** *in our Ndrg4$^{-/-}$ ENS cell cultures, suggesting that Ndrg4 performs a similar function in enteric neurons"*.

c.

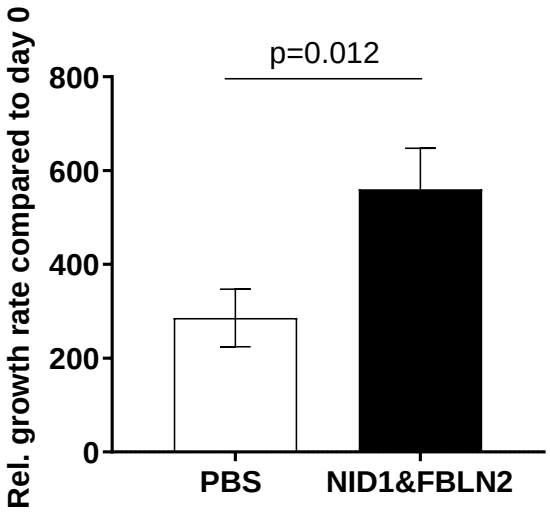
While NID1 and FBLN2 do not have a statistically significant influence on CRC proliferation and migration at every time point, we showed the data at every time point with all p-values in order to give the complete representation of our data.
We now adapted the manuscript text, omitting non-significant data and only describing those reaching statistical significance:
Page 8/9: "Treating cells with NID1&FBLN2 significantly enhanced HCT116 cell proliferation after 72 hours (p=0.044)."
*Page 9: "Whereas the addition of NID1 or FBLN2 did not affect the migration rate of HCT116 cells (**Fig. 5D**), the addition of NID1&FBLN2 had a stimulating effect on HCT116 cell migration after 24 hours (p=0.024). Similarly, after 48 hours of culture, NID1 (p=0.042) and NID1&FBLN2 (p=0.016) had a stimulating effect on the migration velocity of Caco-2 cells compared to the PBS-control condition (**Fig. 5D**)."*.

2. For Figure 4, the authors identified NID1 and FBLN2 as two ECM proteins that are significantly enriched in the secretome of Ndrg4-/- ENS cell cultures (compared to Ndrg4+/+), and found that NID1 and FBLN2 are also highly expressed in the ENS through immunostainings of wildtype tissue sections and Ndrg4+/+ ENS cultures. However, it would also be important to assess NID1 and FBLN2 expression (both at the RNA and protein levels) within Ndrg4-/- tissue sections / ENS cultures, and compare their expression to the levels found in the corresponding Ndrg4+/+ cells.

We have stained intestinal Swiss roll sections of *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice (n=3) with Nid1 and Fbln2 and quantified the intensity (-; -/+, +, ++) of this staining in a blinded manner (2 independent observers). For both proteins this qualitative measurement showed a darker staining in the *Ndrg4*$^{-/-}$ intestine (Nid1 and Fbln2 = 2x ++ and 1x +) than in the *Ndrg4*$^{+/+}$ intestine (Nid1 = 1x + and 2x -/+; Fbln2 = 3x +/-). Thus, these data are in agreement with the identification of higher levels of Nid1 and Fbln2 in the ENS cell secretome of *Ndrg4*$^{-/-}$ ENS cultures and have been added within the manuscript on page 8/9: "*We confirmed the presence of these proteins in the ENS using immunostainings and found specific labeling within both myenteric and submucosal ganglia and interconnecting nerve fibers (**Fig. 4C-E**), **with a stronger intensity of both proteins in the Ndrg4*$^{-/-}$ *intestine than in the Ndrg4*$^{+/+}$ *intestine (Qualitative assessment, data not shown).".***

3. In the section of the paper corresponding to Figure 5, the authors utilized two CRC cell lines (HCT116 and Caco-2) to assess the effect of adding NID1 and/or FBLN2 on their proliferation and migration capabilities. However, the authors identified NID1 and FBLN2 from secretomes of ENS cultures that they showed to have an impact on intestinal epithelial organoid growth rates (Figures 3 and 4). Thus, the authors could also consider testing the effect of NID1 and/or FBLN2 administration on intestinal epithelial organoid growth (in addition to the CRC cell lines), as this will better support their conclusion that these specific factors derived from the ENS secreted fraction can regulate intestinal cell growth.

As suggested by the reviewer, we have examined the combinatorial effect of both recombinant human proteins (commercially available) on human intestinal organoids (HIOs) by adding NID1 and FBLN2 (both in a final concentration of 50µg/ml) to the matrigel dome. This experiment showed that this addition significantly enhanced the relative growth rate of normal human intestinal organoids (**Revision fig. 3** below, p=0.012).



Revision Figure 3. NID1&FBLN2 addition significantly enhances the proliferation rate of HIOs. Addition of NID1&FBLN2 to the matrigel dome significantly enhanced the relative growth rate after five days of culture as compared to the PBS control condition (i.e. similar ratio of PBS to matrigel as NID1&FBLN2 to matrigel). Data are represented as mean ± SEM with the p-value determined using the independent samples T-test (IBM SPSS statistics 25).

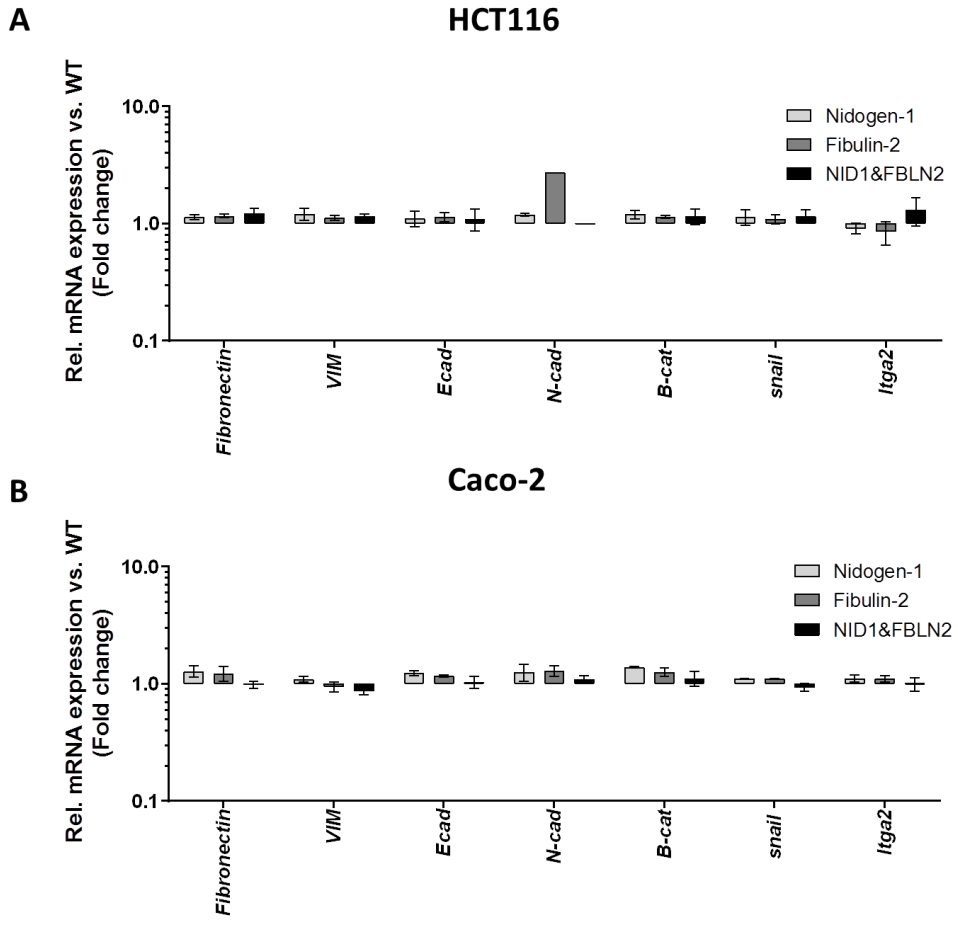
Consequently, we embedded these data within the results section, page 9: *"To explore whether the combinatorial treatment of NID1&FBLN2 affects the proliferation speed of the normal human intestinal epithelium, we exposed normal human intestinal organoids (HIOs) to NID1&FBLN2 within their Matrigel® dome and observed a significantly enhanced relative growth rate of HIOs (Fig. 5B, p=0.012).”*, and within Fig. 5. and its legend (page 25):*“ (B) Addition of NID1&FBLN2 to the Matrigel® dome significantly enhances the relative growth rate of HIOs after five days of culture as compared to the PBS control condition. Data are represented as relative mean percentage ±SEM versus day 0, with the p-value determined using a two-tailed, unpaired t-test.*“. Finally, we also included a further description of this experimental setup within the materials and methods section on page 16: ‘**Primary murine (3D-) cultures**’: *“Primary human intestinal organoids (HIOs) were kindly provided by the group of Dr. T. Wolfs (METC 16-4-185). Similar as described for the murine IEOs, HIO were cultured in 1:1 human OGM/undiluted Matrigel® in the center of a 24-well plate, with refreshment of the medium every two days. Prior to experiments, the HIOs were disaggregated by gentle disruption of the Matrigel® dome with trypsin and incubation for ±2min at 37°C. After pelleting (400g, 5min, 4°C), the HIOs were resuspended in either one of the following conditioned Matrigel® domes: i.e. (1) 1:1 Matrigel®/Human OGM, (2) 1:1 Matrigel®/PBS or (3) 1:1 Matrigel®/NID1/FBLN2 (both at a final concentration of 50µg/ml) to assess the influence of NID1/FBLN2 on the growth rate of HIOs.”* and within the ‘**Co-culture systems and live cell imaging paragraph’** (page 17): *“Similarly, HIO expansion was monitored daily for five consecutive days, starting immediately after solidification of the conditioned Matrigel® dome using the Leica DM3000 microscope (100× magnification). Relative growth rate was determined by measuring the circumference of the HIOs (ImageJ).”*.

4. Given the clinical translatability of the findings from this paper, the authors could better support their results by performing rescue experiments to reintroduce Ndrg4 to Ndrg4-depleted cells and measure effects on cell proliferation and migration.

Unfortunately, it is impossible to perform this experiment within our primary ENS culture system. Consequently, we would have to execute this rescue experiment using cancerous neuronal cell lines, which have a different secretome compared to primary ENS cells, thereby not reflecting the actual physiological situation.

5. In the discussion section, the authors suggest that Ndrg4 knockdown promotes epithelial-mesenchymal transition of intestinal epithelial cells. However, their current set of findings do not directly support this conclusion, as they have only conclusively shown that the Ndrg4-/- cancer mouse models display larger adenomas, and in fact do not display any differences in tumor incidence (initiation). Thus, the authors should revise this statement, or better support their statement by checking for markers of EMT.

As suggested by the reviewer we assessed the RNA expression of different epithelial-mesenchymal transition- (EMT) related genes using RNA isolated from the HCT116 and Caco-2 cells at the end of the migration experiment (Fig. 5C-D within manuscript), but could not detect any statistical significant difference in their expression levels (**Revision Fig. 4A-B**). Consequently, we have removed the following sentence: *"Hence, this suggests that, similar as upon loss of either of its family members (21), knockdown of enteric Ndrg4 stimulates the epithelial-mesenchymal transition (EMT) of intestinal epithelial cells, which is known to enhance tumor progression (21,38,39)."* from the discussion (page 10).



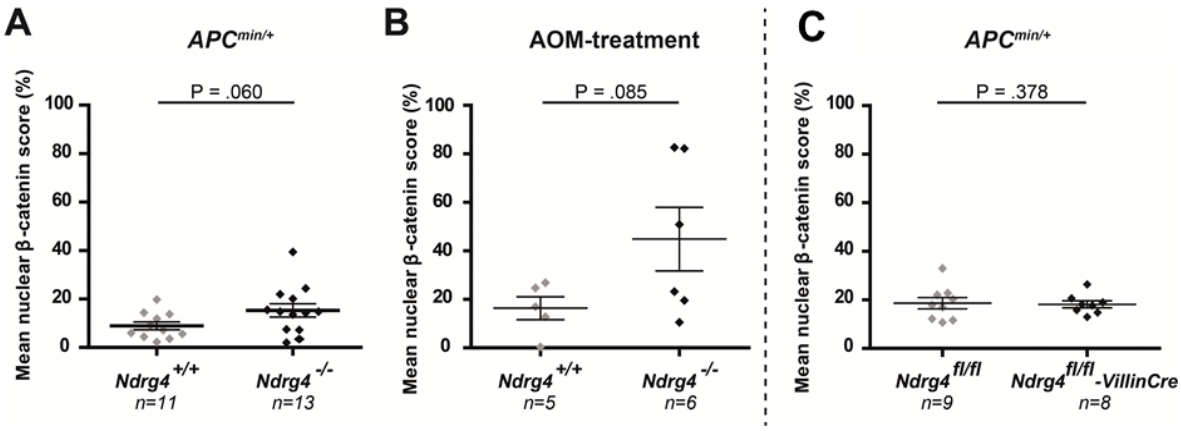
Revision figure 4. Stimulation of CRC cells with NID1 and FBLN2 does not affect the expression of EMT-related genes. qRT-PCR analysis reveals that stimulation of HCT116 (**A**) or Caco-2 (**B**) cells with either NID1, FBLN2 or NID1&FBLN2 does not alter the expression of genes related to the *EMT* (Fibronectin, Vimentin (Vim), E-cadherin (E-Cad), N-Cadherin (N-Cad), β-catenin (β-cat), Snail and integrin alpha 2 (Itga2)) as compared to the PBS-treated cells. Data are derived from two independent experiments and expressed as log10 transformed ± SEM values relative to PBS-treated cells.

Minor points:

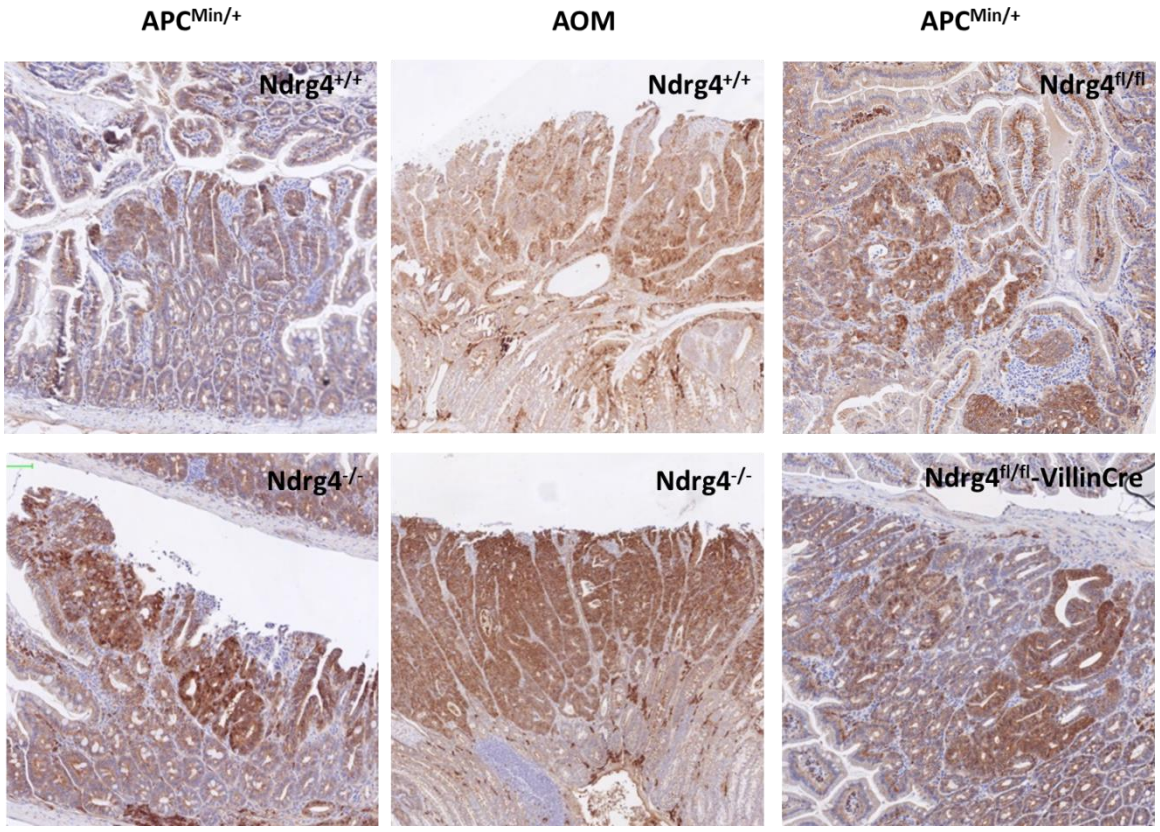
1. In the section of the paper corresponding to Figure 2, the authors describe differences in mean nuclear β-catenin immunoreactivity between adenomas from Ndrg4+/+ and Ndrg4-/- CRC mouse models by comparing some measured values listed in the text. However, it is unclear based on these values how many adenomas were evaluated for each condition, the spread of the data, or how these numbers were obtained (for example, were they normalized to a reference value?). The authors could improve on this result by presenting their data in a graph provided in Figure 2, and support this data with accompanying immunostaining images to better visualize the nuclear localization of β-catenin in these samples.

To define the mean nuclear β-catenin immunoreactivity we have evaluated small intestinal and colonic adenomas in 11 *Ndrg4*$^{+/+}$ and 13 *Ndrg4*$^{-/-}$ mice (*Apc*$^{Min/+}$ model) and 5 *Ndrg4*$^{+/+}$ and 6 *Ndrg4*$^{-/-}$ mice (AOM-treatment model). In these models, we have evaluated on average 11 small intestinal and 1.5 colonic adenomas per mouse, respectively. In addition, we also evaluated the nuclear positivity in small intestinal adenomas (about 8 per mouse) in the *Ndrg4*$^{fl/fl}$ (n=8) and *Ndrg4*$^{fl/fl}$*-VillinCre* (n=9) mice (*Apc*$^{Min/+}$ model). The data have not been normalized to a reference value, but we performed a semi-quantitative scoring based on the methods described by Chung et al (2001) and Wong et al (2004), in a blinded manner. Detailed description of the scoring method: the intensity of β-catenin positivity (0=no expression, 1=weak expression, 2=moderate expression, 3=strong expression, 4=very strong expression) was determined separately in the membrane, cytoplasm and nucleus for each intestinal tumor. The mean nuclear β-catenin score was expressed as the percentage of cells with a positively stained nucleus multiplied by the staining intensity (described within the Supplementary Materials and Methods, Morphometric analysis and intestinal histopathology).

Based on previous reviewer's comments and the fact that this scoring is not statistically significantly higher in the *Ndrg4*$^{-/-}$ compared to the *Ndrg4*$^{+/+}$ mice (as depicted in major comment 1), we decided to omit these graphs from Fig. 2 within the manuscript and only focus on these results in the text by describing the score as "mean ± standard error" and corresponding p-value. Nonetheless, for clarity, **Revision fig. 5** below depicts the results and the spreading of the data. In addition, **Revision fig. 5** depicts representative images of the β-catenin staining.



Revision figure 5. Mean nuclear β-catenin scoring in the three CRC models. (**A-B**) Adenomas in the intestinal tract of *Ndrg4*$^{-/-}$ mice have a non-statistically significantly higher nuclear β-catenin content than intestinal adenomas of *Ndrg4*$^{+/+}$ mice. (**C**) In contrast, *NDRG4*$^{fl/fl}$ and *NDRG4*$^{fl/fl}$-*VillinCre* mice harbor adenomas with an equivalent mean nuclear β-catenin content. Data are represented as mean ± SEM and p-values calculated using a two-tailed, unpaired t-test (IBM SPSS statistics 25).



Revision figure 6. Representative images (20x magnification) of the β-catenin staining in the three CRC mouse models.

2. In Figure 3D, the authors present their RNA expression analysis for a number of genes that were downregulated as a result of Ndrg4 knockdown in ENS cultures. These hits could be further validated by protein expression assays such as western blots or immunostainings. Moreover, given that the

identified hits suggest a role for Ndrg4 in vesicle transport, an immunostaining analysis could also reveal changes in subcellular localization, apart from changes in overall expression levels.

We have explored the expression of conventional vesicle markers (e.g. phogrin, synapsin) and of neuropeptides with a varicose localization pattern (e.g. vasoactive intestinal peptide, substance P) using confocal microscopy but did not observe apparent differences between *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ tissues. A detailed study focusing on the effect of *Ndrg4* deficiency on the subcellular localization of members of the vesicle trafficking machinery probably requires a dedicated electron microscopy study, which we feel is outside the scope of the current manuscript.

3. The reference to Figure 5C in the sentence "As shown in Fig. 5C, the singular addition of either NID1 or FBLN2 did not affect the proliferation rate of HCT116 cells..." was wrongly indicated, the corresponding figure for this result should be Figure 5B.

According the referee's comments above (major comment 1c), this sentence has been removed from the manuscript.

1st Revision - Editorial Decision 22nd Jan 2021

Dear Dr. Melotte,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees support the publication of your study in EMBO reports. However, all referees have remaining concerns and suggestions to improve the manuscript, we ask you to address in a final revised manuscript. Please also provide a detailed point-by-point response addressing these issues.

Moreover, I have these editorial requests I also ask you to address in a final revised manuscript:

- Please provide the abstract written in present tense.

- Please remove the paragraph 'significance' from the manuscript or fuse it with the abstract. Please make sure that the final abstract has not more than 175 words.

- We would like to publish your manuscript as Report (as there are only 5 figures). For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- Please move all the methods information form the Appendix to the main manuscript. The Appendix Table could be fused with Table 1.

- It seems that authors Remond Fijneman and Robert Hofstra are missing from the author contributions. Please check.

- Per journal policy, we do not allow 'data not shown' (see pages 6 and 9 of your manuscript). All data referred to in the paper should be displayed in the main or Expanded View figures, or an Appendix. Thus, please add these data, or (if these are not essential) remove the 'data not shown'. See:
http://www.embopress.org/page/journal/14693178/authorguide#unpublisheddata

- The Western blots shown in Fig. 3 (and the source data file) are over-contrasted. Please show the blots in the manuscript as unmodified as possible (and with similar contrast and brightness as in the source data).

- Please also note our new reference format. Please format your reference list accordingly:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends. Please provide error bars and statistical testing where applicable.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to

address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to four bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

The quantifications of submucosal cell bodies and innervation of villi are satisfactory and provide further proof of the claim that the ENS is not largely affected in Ndrg4 mutant mice. Other major concerns have also been sufficiently answered. The study provides novel insights into the role of the ENS in intestinal cancers, a poorly explored research area. It is likely to prime further mechanistic investigation and places the ENS as a new potential cancer treatment target.

Minor Issues:

Abstract: "ENS" is used before "Enteric Nervous System" is correlated to this abbreviation.

Reference 33: Refer to the online site and paper, and put this reference after the original paper, ie swop Reference 33 and 34.
It could read:
Online Resource http://mousebrain.org/; original paper: Zeisel A, et al., Cell 2018; 174:999-1014.

Referee #2:

Whilst the authors have made some good faith efforts to revise the manuscript in accordance with reviewer comments, a number of issues remain:
1) I am surprised that the authors find it acceptable to retain data this is not statistically significant. The proliferation data for the CRC lines is not very impressive when the statistically insignificant time-points are removed.
2)The experiment suggested by reviewer 2, which is to cross the Ndrg4-/- mice with a ENS-Cre line would very much help to determine the acute effects of losing Ndrg4 expression on tumour growth (proliferation etc). Failing this, can the authors reproduce the growth promoting effects of recombinant Nid1/Fbln2 seen with normal intestinal organoids on colon cancer organoids generated from their APCmim/AOM mice?

3) The Lgr5 IHC in Fig 1 is very poor and does not reflect the known endogenous expression pattern in the colon. Since Lgr5 antibodies are notoriously poor, effects on the stem cell compartment should be evaluated for the SI and Colon using either standard ISH for OLFM4 or RNAscope for Lgr5.

Referee #3:

The revised manuscript has been improved and most initial points are addressed.
Only one minor point is, regarding to my original comment " Are there any expression within adenoma?", it would be appreciated if the authors can show fiburin and nidogen IHC images of adenoma/tumor area. They need to confirm whether tumor cells or tumor stroma start to express these proteins, and whether fiburin/nidogen expression at myoenteric plexus near the tumor area is increased compared to the non-tumor, normal area.

We would like to thank the reviewers and editor for their positive appreciation of our work and constructive remarks, which have further improved our manuscript. We corrected and addressed the remaining issues.

Referee #1:

The quantifications of submucosal cell bodies and innervation of villi are satisfactory and provide further proof of the claim that the ENS is not largely affected in Ndrg4 mutant mice. Other major concerns have also been sufficiently answered. The study provides novel insights into the role of the ENS in intestinal cancers, a poorly explored research area. It is likely to prime further mechanistic investigation and places the ENS as a new potential cancer treatment target.

Minor Issues:

1. Abstract: "ENS" is used before "Enteric Nervous System" is correlated to this abbreviation.

This has been corrected (page 3).

2. Reference 33: Refer to the online site and paper, and put this reference after the original paper, i.e. swop Reference 33 and 34. It could read: Online Resource http://mousebrain.org/; original paper: Zeisel A, et al., Cell 2018; 174:999-1014.

This has been adapted accordingly within the manuscript on page 10.

Referee #2:

Whilst the authors have made some good faith efforts to revise the manuscript in accordance with reviewer comments, a number of issues remain:

1) I am surprised that the authors find it acceptable to retain data this is not statistically significant. The proliferation data for the CRC lines is not very impressive when the statistically insignificant time-points are removed

Even though we initially retained the non-significant data to give a complete representation of our data, as requested by the reviewer, we now removed the single treatment results on CRC cell proliferation and migration. Consequently, we have adapted panel C and D within Figure 5 of the manuscript and also revised the manuscript text on page 11-12 accordingly.

2) The experiment suggested by reviewer 2, which is to cross the Ndrg4-/- mice with a ENS-Cre line would very much help to determine the acute effects of losing Ndrg4 expression on tumor growth (proliferation etc). Failing this, can the authors reproduce the growth promoting effects of recombinant Nid1/Fbln2 seen with normal intestinal organoids on colon cancer organoids generated from their APCmim/AOM mice?

We agree with the reviewer that the use of an ENS-specific Cre line would strengthen our findings. However, we do not have such a model available within our institute. Also, when transferring our manuscript to EMBO reports, the editor pointed out that these experiments would not be required. Although the experiment to collect organoids from $APC^{min/+}$/AOM mice is an interesting suggestion, no recombinant murine Nid1 and/or Fbln2 is commercially available to perform these experiments.

3) The Lgr5 IHC in Fig 1 is very poor and does not reflect the known endogenous expression pattern in the colon. Since Lgr5 antibodies are notoriously poor, effects on the stem cell compartment should be evaluated for the SI and Colon using either standard ISH for OLFM4 or RNAscope for Lgr5.

Based on the previous suggestion by the reviewer to check the stem cell compartment using either Lgr5 or OLFM4, we stained for Lgr5 using the Anti-Lgr5 antibody (Ab71225, Abcam) available within our institute. We agree with the reviewer that there is quite some background staining using this Lgr5 antibody, and therefore omitted the images from panel A within Figure 1 of the manuscript. However, a negative control without any positivity (omission Lgr5 primary antibody), a similar number of stem cells (Lgr5 or OLFM4 positive)/crypt as depicted in other studies (Dehmer JJ et al, PLoS One 2011; Fernandez-Vallone V et al, EMBO Rep 2020), and extensive evaluation by our animal pathologist (MJG), support the adequate representation of the Lgr5 population. The results are now added within Expanded View Figure 1D-E.

Referee #3:

The revised manuscript has been improved and most initial points are addressed.
Only one minor point is, regarding to my original comment " Are there any expression within adenoma?", it would be appreciated if the authors can show fibulin and nidogen IHC images of adenoma/tumor area. They need to confirm whether tumor cells or tumor stroma start to express these proteins, and whether fibulin/nidogen expression at myenteric plexus near the tumor area is increased compared to the non-tumor, normal area.

To address this question, we have stained normal colon, adenoma and carcinoma tissue (MPTC numbers 2011-10 and 2015-12) with NID1 and FBLN2 as described within the materials and methods section, immunohistochemistry (page 17-18). These data reveal that the expression of NID1 and FBLN2 within the myenteric plexus did not increase in adenoma/tumor tissue compared to normal tissue. However, while only limited expression was observed in normal epithelium, increased expression of both proteins was observed in tumor cells/tumor stroma. Consequently, we have added these data within the manuscript text (page 12) and in panel F of Figure 5.

2nd Revision - Editorial Decision 25th Feb 2021

Dear Dr. Melotte,

Thank you for the submission of your final revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Referee #2 has a final concern/suggestion we ask you to address by appropriate text changes in a further revised version. I do not think the claim should be removed. But please discuss this point in more detail in the manuscript. Please also provide a final response to this referee with your revised manuscript.

Moreover, I have these further editorial requests I ask you to also address:

- Please remove the bullet points, the synopsis blurb and the synopsis image from the final manuscript text. I have saved these separately and will forward them to our publisher upon acceptance.

- For the synopsis image, please provide this in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels. Please make sure that in the final version in this size all fonts remain legible.

- several rather bright microscopic images also have white scale bars, that are often difficult to see. Please provide these images (with whit/bright background) with black scale bars.

- In the legend of the EV figures, both figures are named EV2. Please check.

- Finally, we require that primary datasets produced in this study (i.e. the mass spec data) are deposited in an appropriate public database. Please deposit the data and list the accession numbers and database in the "Data Availability" section. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

See also: http://embor.embopress.org/authorguide#datadeposition

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim

Achim Breiling
Editor
EMBO Reports

Referee #2:

The authors have not convincingly shown that there is no effect on stem cells and this claim should

be removed.

I find the argument that the requisite Cre line is not available in the Institute very weak - this should have been considered in advance and appropriate mouse lines imported if necessary. However, if the editors agree that it is not necessary, then I have nothing further to add.

Referee #3:

The manuscript is suitable for publication in EMBO reports without revision.

We would like to thank the reviewers and editor again for their constructive and positive remarks.

Editorial requests:

- Please remove the bullet points, the synopsis blurb and the synopsis image from the final manuscript text. I have saved these separately and will forward them to our publisher upon acceptance
- For the synopsis image, please provide this in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels. Please make sure that in the final version in this size all fonts remain legible.
- Several rather bright microscopic images also have white scale bars, that are often difficult to see. Please provide these images (with whit/bright background) with black scale bars.
- In the legend of the EV figures, both figures are named EV2. Please check.

  We have addressed all the editorial request above within the final manuscript text and final figure files.

- Finally, we require that primary datasets produced in this study (i.e. the mass spec data) are deposited in an appropriate public database. Please deposit the data and list the accession numbers and database in the "Data Availability" section. Please note that the Data Availability Section is restricted to new primary data that are part of this study. See also: http://embor.embopress.org/authorguide#datadeposition

  We have deposited the mass spectrometry data of the murine ENS secretome via the PRIDE partner repository in the ProteomeXchange Consortium, with the identifier PXD024502, which is also described within the “Data availability” section. The following username and password apply to this dataset: reviewer_pxd024502@ebi.ac.uk and hMRD2m3S, respectively.

  Human proteomics data of NID1 and FBLN2 are attached to this manuscript in a source data excel and PDF file: “Source proteomics data NID1&FBLN2 human normal and CRC secretome” and “Source annotated MSMS spectra NID1&FBLN2”, respectively. The entire dataset is still under embargo as they are currently in the process of finalizing the findings observed within this dataset. As soon as the entire dataset is made publically available, we will also provide the accession number of the entire database.

Referee #2:

1) The authors have not convincingly shown that there is no effect on stem cells and this claim should be removed.

As agreed by the editor we did not remove this claim, but we discussed this point in more detail within our manuscript (page 6): “*The numbers of Lgr5-positive cells detected within small and large intestinal segments (**Fig. EV1D-E**) were similar as previously described (Dehmer et al, 2011; Fernandez Vallone et al, 2020). However, no differences in the number of Lgr5-positive cells were observed between Ndrg4$^{+/+}$ and Ndrg4$^{-/-}$ intestines*“.

2) I find the argument that the requisite Cre line is not available in the Institute very weak - this should have been considered in advance and appropriate mouse lines imported if necessary. However, if the editors agree that it is not necessary, then I have nothing further to add.

As the establishment of this mouse model within our institute would take a considerable amount of time, it was discussed with the editor after the first review process (EMBO Journal) to exclude these experiments from the revisions. We thank the reviewer for their understanding.

Referee #3:

The manuscript is suitable for publication in EMBO reports without revision.

3rd Revision - Editorial Decision 11th Mar 2021

Veerle Melotte
Maastricht University Medical Center and Erasmus University Medical Center
Netherlands

Dear Dr. Melotte,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

IMPORTANT: Please make sure that the PRIDE dataset is public latest the day the paper is published online.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Veerle Melotte
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51913V4

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	A power calculation was performed prior to designing the experimental set up.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The approprate sample size was based on previous experience and previous experiments from our collaborators and further strengthened using a power-calculation.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Only for the whole-gut transit experiment, we had a pre-defined exclusion criterion: i.e. "Maximum observation time was five hours. Mice who failed to expel a pellet were noted with a maximum transit time of 300 min."
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals were age- and gender matched and randomly housed in groups of 3 to 5 under standard conditions having free access to food and water. For all analyses, every researcher involved was blinded to the genotype for animal experiments and blinded to the experimental conditions in vitro.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were age- and gender matched and randomly housed in groups of 3 to 5 under standard conditions having free access to food and water.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For all analyses, every researcher involved was blinded to the genotype for animal experiments and blinded to the experimental conditions in vitro.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animals were age- and gender matched and randomly housed in groups of 3 to 5 under standard conditions. Researchers were blinded to the genotype during the experiments and analysis.
5. For every figure, are statistical tests justified as appropriate?	Yes, described within the statistical analysis section and described within every figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Shapiro-wilk test for normality; F-test for variances
Is there an estimate of variation within each group of data?	SEM is shown in every figure containing multiple experimental data points.

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http://www.consort-statement.org
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Is the variance similar between the groups that are being statistically compared?	To assess whether the variances were equal, an F-test for variances was applied. Based on this outcome, the appropriate p-value was used.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All necessary information regarding antibodies has been provided within the (Supplementary) materials and methods sections. Prior to use, we have internally checked the validity of the antibodies according their sensitvity and specificity (conform criteria defined by Pradidarsheep et al, J Histochem Cytochem, 2008).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	CRC cells derived from LGC, Teddington, UK and profiled using STR-DNA typing by the Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell cultures, Germany (August 2019).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Ndrg4 wild-type (Ndrg4+/+) and knock-out (Ndrg4-/-) littermates, and Ndrg4 floxed (Ndrg4fl/fl) mice, kindly provided by Prof. Baldwin (Vanderbilt University Medical Centre, Nashville, USA[55]), were on a mixed C57BL/6 genetic background (matching 59.7% C57BL/6NRj and 66.8% C57BL/6NRj, respectively; GVG Genetic Monitoring, Germany). VillinCre mice on the C57BL/6J genetic background (intestinal epithelial-specific Cre model) were kindly provided by Prof. Köhler (Maastricht University, Maastricht, The Netherlands). APCMin/+ mice, also on a C57BL/6J background, which have a genetic predisposition to intestinal adenoma formation, were purchased from the Jackson Laboratory (002020, Bar Harbor, USA). Prior to experiments, all mice were characterized by genotyping PCR [22], using the primer sequences listed in Table 1. Animals were age- and gender matched and randomly housed in groups of 3 to 5 under standard conditions having free access to food and water. Both males and females were included within this study.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Collection, storage and use of tissue and patient data were performed in compliance with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (https://www.federa.org/). METC approval(16-4-185) for experiments using humin intestinal organoids. All animal experiments were approved by the Committee of Animal Welfare of Maastricht University and performed according to Dutch regulations.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	To our knowledge we have adhered to the appropriate guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Adherence to: "Code for Proper Secondary Use of Human Tissue in the Netherlands" (https://www.federa.org/). Approval by the Medisch-ethische toetsingscommissie azM/UM (METC azM/UM) for use of human material.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The mass spectrometry proteomics of the murine ENS secretome have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol Y, 2019) partner repository with the dataset identifier PXD024502. Human proteomics data of NID1 and FBLN2 are attached to this manuscript in a source data excel and PDF file: "Source proteomics data NID1&FBLN2 human normal and CRC secretome" and "Source annotated MSMS spectra NID1&FBLN2", respectively.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA