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# Differential Notch and TGF$\beta$ signaling in primary colorectal tumors and their corresponding metastases

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**Abstract.** *Background:* Loss of epithelial morphology and the acquisition of mesenchymal characteristics may contribute to metastasis formation during colorectal tumorigenesis. The Wnt, Notch and TGF$\beta$ signaling pathways control tissue homeostasis and tumor development in the gut. The relationship between the activity of these pathways and the expression of epithelial and mesenchymal markers was investigated in a series of primary colorectal tumors and their corresponding metastases. *Methods:* Tissue samples of primary colorectal tumors, normal colonic mucosa, and regional and systemic metastases were processed for immunohistochemistry in a tissue microarray format. The expression of mesenchymal (vimentin, fibronectin) and epithelial (E-cadherin) markers was related to markers of Wnt ($\beta$-catenin), Notch (HES1) and TGF$\beta$ (phospho-SMAD2) signalling. In addition, the KRAS mutation status was assessed. *Results:* When compared to normal mucosa, primary colorectal tumors showed a marked increase in the levels of cytoplasmic vimentin and nuclear $\beta$-catenin, phospho-SMAD2 and HES1. Increased vimentin expression correlated with the presence of oncogenic KRAS and with nuclear $\beta$-catenin. The corresponding liver, lymph node, brain and lung metastases did not express vimentin and displayed significantly lower levels of nuclear phospho-SMAD2 and HES1, while retaining nuclear $\beta$-catenin. *Conclusions:* Primary colorectal carcinomas display aberrant expression of vimentin, and have activated Notch and TGF$\beta$ signaling pathways. Surprisingly, many regional and distant metastases have lost nuclear HES1 and pSMAD2, suggesting that the activity of the Notch and TGF$\beta$ pathways is reduced in secondary colorectal tumors.

Keywords: Notch, vimentin, HES1, colorectal, metastasis, epithelial mesenchymal transition, KRAS

## 1. Introduction

Colorectal cancer is one of the most common malignancies in the Western world. It is now well established that the development of colorectal cancer results from dysregulation of signaling pathways that normally control homeostasis in the gut, in particular those activated by the Wnt, Delta/Jagged and TGF$\beta$ ligands [1]. In addition, mutational activation of the KRAS oncogene contributes to the initiation and/or progression of colorectal cancer [2]. These pathways do not operate as single independent entities, but extensive cross-talk and cooperation is required for tumor formation. In addition to the specific mutations and deletions that cause activation of the above signaling pathways, the majority of colorectal tumors is characterized by gross chromosomal instability [3]. Furthermore, specific chromosomal aberrations and gene expression profiles are associated with the ability of colorectal tumors to metastasize [4–6].

Approximately 50% of colorectal cancer patients develop metastatic disease. The mechanisms underlying metastasis formation are the subject of intense study. It has been proposed that the acquisition of mesenchymal properties allows epithelial tumor cells to detach from the primary tumor mass, invade and migrate to distant sites [7,8]. This process, which shares some features with the epithelial-mesenchymal transitions (EMT) that occur during embryogenesis, is controlled by the same signaling pathways that promote initial tumor formation [9]. However, metastases from colorectal tumors retain their epithelial phenotype, suggesting that metastatic tumor cells most likely

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do not undergo true EMT. Vimentin is an intermediate filament protein normally expressed in mesenchymal cells [10] and is the most well established marker of mesenchymal differentiation in human tumors. Accumulating evidence suggests that the aberrant expression of vimentin in epithelial cancer cells is related to local invasiveness and metastatic potential [11–14].

The aim of this study was to investigate whether the Wnt, Notch, TGF$\beta$ and KRAS pathways are differentially active in primary tumors versus regional and distant metastases and whether this is accompanied by changes in the expression of established markers of epithelial and mesenchymal differentiation.

## 2. Material and methods

### *2.1. Patients and materials*

Patients with liver metastases of colorectal carcinoma treated at the University Medical Center Utrecht, the Antonius Hospital in Nieuwegein or the Diakonessenhuis in Utrecht between 1992 and 2004 were identified retrospectively. Samples of normal colon, primary colorectal carcinoma and their corresponding liver metastases were obtained during surgery from 61 patients. Paraffin blocks of metastatic lymph nodes from 4 patients were also available for study. There were 40 males and 21 females with a mean age of 62.7 years (range 40–77 years) at time of surgery for liver metastases.

A second group of 14 patients [10 male, 4 female, mean age 54.4 years (range 37–76 years)] who underwent resection of their primary colorectal carcinoma at the University Medical Center Utrecht between 1995 and 1998 and who had remained disease-free for at least 5 years following resection were also identified. Samples of normal colon and colorectal carcinoma were obtained from all these patients.

Furthermore, samples of 5 colorectal lung metastases and 6 colorectal brain metastases were available for study.

Anonymous use of left over resection specimens for scientific purpose is part of the standard treatment agreement with patients in our hospital (opt-out system) [15]. The research proposal was approved by the scientific committee of the Department of Pathology.

### *2.2. Preparation of tissue micro arrays*

A tissue microarray (TMA) was constructed as described before [16]. In brief, fresh 4 μm sections were obtained from the selected paraffin-embedded tumor blocks and stained with haematoxylin and eosin (H&E). Non-necrotic, representative areas of tumor specimens or normal tissue were marked on the H&E slide. Core needle biopsies were retrieved from the original tumor blocks (marked areas) using a manual tissue array instrument (Beecher Instruments, Sun Prairie, WI, USA) and positioned in a recipient paraffin array block. Three core biopsies from each sample were obtained.

### *2.3. Immunohistochemical analysis*

Immunohistochemical staining was performed using standard methods. Sections of 4-μm were cut from the TMA, deparaffinized in xylene, followed by rinsing in graded ethanol and dehydrated in distilled water. After incubation with 3% hydrogen peroxide for 20 min, antigen retrieval was achieved by boiling in 10 mM citrate buffer pH 6.0 for 10 min or 1 mM EDTA buffer (pH 9.0) and sections were washed with TBS. For staining with E-cadherin, $\beta$-catenin and vimentin an automated immunostainer (MARK5, DPC, Breda, The Netherlands) was used. For staining with HES1 sections were blocked with 5% goat serum in TBS for 1 h, washed with TBS, and incubated at 4°C overnight. All primary antibodies used were commercially available and well-validated: E-cadherin (1:200; Invitrogen Corporation, Carlbad, CA, USA), $\beta$-catenin (1:1600, Becton–Dickinson, Franklin Lakes, NJ, USA), vimentin (1:400, Dako, Glastrup, Denmark), HES1 (1:300, Chemicon, Temucula, CA, USA) fibronectin (1:200), p-SMAD2/3 (1:200, Cell Signaling Technology, Danvers, MA, USA). HRP-conjugated secondary antibodies (DPVM-55HRP or DPVM-110HRP, Immunologic, Duiven, The Netherlands) were detected with 3.3′-diaminobenzidine substrate (D4418, Sigma, Saint Louis, USA). Slides were counterstained with heamatoxylin and rinsed with water, dehydrated in graded ethanol, cleared in xylene and coverslipped. Appropriate positive and negative controls were used in all experiments.

### *2.4. DNA isolation*

Genomic DNA was extracted from paraffin-embedded tumor specimens. Ten to twelve serial sections of 10 μm and one of 4 μm were cut from the tissue blocks. The 4 μm section was stained with haematoxylin and eosin for exact identification and delineation of tumor areas. The 10 μm sections were de-

paraffinized with xylene and rehydrated by washing with 100% ethanol, 95% ethanol, 70% ethanol and distilled water, and were dried. The indicated tumor areas were collected by microdissection. The dissected tissue was suspended in extraction buffer (1 M Tris, 0.5 M EDTA and 10% sodium dodecyl sulphate) containing proteinase K (1 mg/ml, Roche) and incubated at 56°C for 48 hours. Proteinase K was freshly added every 12 hours. After heat inactivation of proteinase K, NaCl was added to a final concentration of 0.4 M and the solution was extracted twice with 25:24:1 mixture of phenol–chloroform–isoamylalcohol. Genomic DNA was precipitated with ethanol, pelleted and subsequently resuspended in TE buffer (Tris-HCL 10 mM, EDTA 1 mM). The concentration of DNA was measured by optical densitometry.

### 2.5. Detection of Kras mutations, PCR amplification and sequencing

Mutation in codon 12 and 13 of the Kras gene were detected by nested PCR. The first PCR was performed with gene-specific primers (forward 5′-TTCATTACGATACACGTCTGC; reverse 5′- GAAA CCCAAGGTACATTTCAG) and carried out at 35 cycles each of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min, followed by a final elongation for 2 min at 72°C. PCR reaction mixes contained 5 μl genomic DNA (10 ng/μl), 0.2 μM forward primer, 0.2 μM reverse primer, 200 μM of each dNTP, 24 mM Tricine, 8.0% Glycerol (w/v), 1.6% DMSO (w/v), 2 mM $MgCl_2$, 85 mM Amonium acetate (pH 8.7), and 0.2 U Taq polymerase in a total volume of 10 μl. Of the first PCR reaction product, 1 μl was used as template for the nested PCR reaction. The second PCR reaction contained gene-specific primers (forward 5′-TG TAAAACGACGGCCAGTACTGGTGGAGTATTTG ATAGTG; reverse 5′-AGGAAACAGCTATGACCAT ATCAAAGAATGGTCCTGCAC). The PCR reaction mixes contained 0.2 μM forward primer, 0.2 μM reverse primer, 200 μM of each dNTP, 24 mM Tricine, 8.0% Glycerol (w/v), 1.6% DMSO (w/v), 2 mM $MgCl_2$, 85 mM Amonium acetate (pH 8.7), and 0.2 U Taq polymerase in a total volume of 10 μl. Cycling conditions were the same as for the first PCR reactions. Nested PCR products were diluted with 20 μl distilled water and 1 μl was used as template for the sequencing reactions. Constructs were verified by automatic sequencing using BigDye Terminator v1.1 (Applied Biosystem, Warrington, UK). The samples were purified by Sephadex Superfine 75 column and analyzed on an ABI PRISM fluorescent automated DNA sequencer for point mutations.

### 2.6. Evaluation of immunostaining

Two investigators (L.V., P.vD.) simultaneously assessed the results of immunostaining without the knowledge of the patients' clinicopathological details. Single assessment of the intensity of staining only was performed for those markers showing very homogeneous staining, for instance vimentin. In the few sections that showed non-homogeneous staining, the highest score was taken. For markers that did not show homogeneous staining throughout the section (for instance HES1 and pSMAD2/4), we analyzed % positive nuclei.

Cytoplasmic, membraneus and nuclear staining were evaluated as absent, weak, strong or very strong. Nuclear HES1 and pSmad 2/4 staining were evaluated as percentage. The mean scores of 3 needle biopsies per tumor were plotted. The inter-observer quality of evaluation was generally very good (80%). Sections that were evaluated differently were re-examined to reach a consensus. The criteria for evaluation of membrane and nuclear staining were: Membranous: absent, weak, strong. Nuclear: for b-catenin: absent, weak, strong (homogeneous staining intensity within the sections). Nuclear: for HES1 en Smad 2/4: % positive cells (non-homogeneous staining intensity within the sections).

### 2.7. Statistical analysis

Statistical analysis was performed with GraphPad Prism™ version 3.0 for Windows (GraphPad Software, San Diego, California, USA). Statistical differences between groups were analyzed by Mann–Whitney $U$ test and $p < 0.05$ (two tailed) was used to denote statistical significance. Values are expressed as mean ± SEM.

## 3. Results

### 3.1. Expression of vimentin in primary colorectal tumors but not in metastases

Normal mucosal epithelial cells did not express the mesenchymal marker vimentin, as expected. To our surprise, primary colorectal tumors expressed vimentin at varying levels (Fig. 1A and B) but this expression was completely lost in the corresponding liver metastases ($p < 0.001$). Similarly, metastases in lymph nodes ($n = 4$), lung ($n = 5$) and

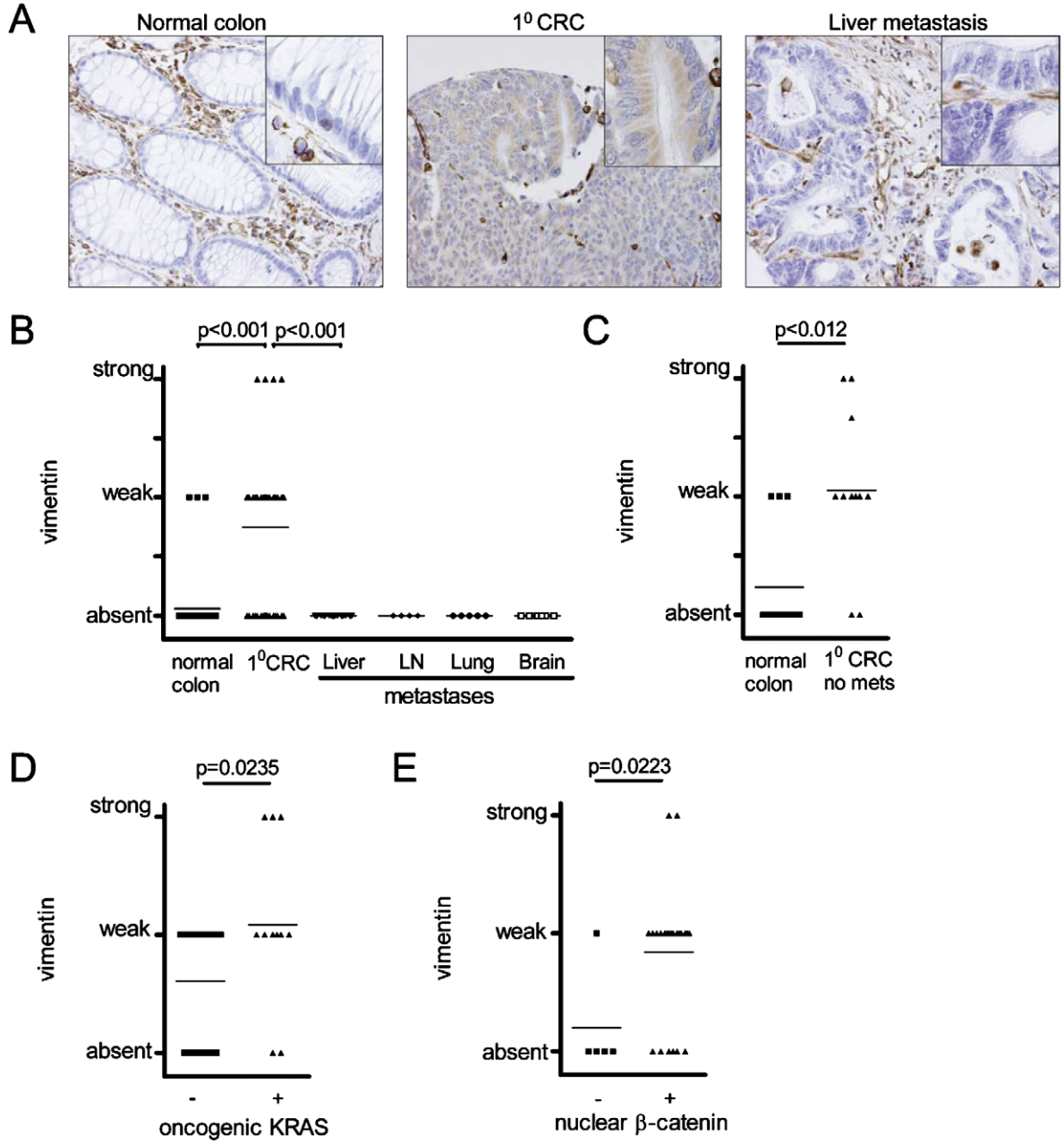


Fig. 1. Expression of vimentin in primary CRC. (A) In normal colon epithelium vimentin is highly expressed in stromal fibroblasts but not in epithelial cells surrounding the lumen. In primary colorectal tumor samples the tumor cells express moderate levels of cytoplasmic vimentin in a homogeneous staining pattern. Also in these samples the stromal cells express high levels of vimentin. Vimentin was not expressed in the tumor cells of the corresponding liver and lymph node metastasis, whereas stromal cells in these tumors were highly positive. Magnification 20×. Inset 40×. (B) Scatter diagram demonstrating expression of vimentin in primary colorectal carcinoma, normal colon epithelium and the corresponding metastases in liver lymph nodes (LN), lung and brain. (C) Vimentin expression in normal colon epithelium and in colorectal carcinoma of patients without liver metastasis. Vimentin expression was significantly higher in colorectal carcinoma tissue compared to normal colon epithelium, $p = 0.012$. (D) Scatter diagrams demonstrating that the expression of vimentin in primary CRC correlates with the presence of oncogenic KRAS and (E) nuclear $\beta$-catenin.

brain ($n = 6$) were completely negative for vimentin (Fig. 1A and B). Non-metastatic primary colorectal tumors expressed vimentin comparable to metastatic tumors (Fig. 1C). Thus, vimentin was expressed in the majority of metastatic and non-metastatic primary colorectal tumors, but expression was lost in regional and distant metastases. The observed changes in vimentin expression were not associated with obvious EMT-like morphological changes of the tumor phenotypes (not shown). None of the primary tumors or their metastases expressed fibronectin (not shown).

### 3.2. Vimentin expression in primary CRC is associated with the presence of oncogenic KRAS and with nuclear β-catenin

Mutational activation of the KRAS proto-oncogene is a critical event in the initiation and progression of colorectal cancer and metastasis formation [17,18]. Furthermore, Ras signaling promotes EMT in in vitro cell systems [19]. Therefore, we evaluated whether the presence of oncogenic KRAS in primary colorectal tumors was associated with vimentin expression. Mutations in the KRAS gene in primary colorectal tumors were found in 18 (30.5%) of 59 patients. Mutations in codon 12 and 13 were detected in 9 cases each. Mutation analysis failed in 2 patient samples. Tumors expressing oncogenic KRAS expressed significantly higher levels of vimentin ($p = 0.024$, Fig. 1D).

Activation of Wnt signalling leads to nuclear accumulation of β-catenin and is a key event in the development of the majority of colorectal tumors [20]. In our series the majority, but not all, of the primary colorectal tumors displayed accumulation of nuclear β-catenin (see below). Interestingly, the few tumors that did not show nuclear β-catenin ($n = 5$) expressed significantly lower levels of vimentin (Fig. 1E). Thus, vimentin expression in primary colorectal tumors is associated with the presence of oncogenic KRAS and with accumulation of nuclear β-catenin.

### 3.3. Membranous E-cadherin in primary CRC and liver metastases

E-cadherin is essential for maintaining proper cell–cell contacts and its loss contributes to tumor development and metastasis formation [21]. Therefore, we evaluated expression and localization of E-cadherin in normal mucosa, primary metastatic colorectal tumors and their liver metastases. A minority of primary tumors (13/57) and metastases (11/51) displayed reduced membranous localization of E-cadherin. Thus, the majority of the tumors retained strong membranous E-cadherin staining which was not significantly different from that observed in normal mucosa (Fig. 2). Furthermore, the morphological differentiation status of primary tumors and their corresponding metastases was not significantly different (not shown). Taken together, the results show that primary colorectal cancers express the mesenchymal marker vimentin without undergoing gross loss of the epithelial phenotype.

### 3.4. Increased Wnt signaling in primary colorectal tumors and in metastases

Next, we investigated whether signaling pathways known to control colorectal cancer development and epithelial (de-)differentiation, were associated with vimentin expression. Aberrant Wnt signaling leads to nuclear accumulation of β-catenin which contributes to initiation of colorectal tumor formation. Normal colonic epithelium showed localization of β-catenin at the membrane, but not in the cytoplasm or in the nucleus (Fig. 3A–D). However, β-catenin was found in the cytoplasm and in the nucleus of the vast majority of primary metastatic colorectal tumors and the corresponding liver metastases, which indicates activation of the Wnt signaling pathway in the majority of these tumors (Fig. 3A–D), as expected.

### 3.5. Increased TGFβ signaling in primary colorectal tumors but not in metastases

TGFβ signaling suppresses the early development of colorectal tumors but can stimulate tumor progression at later stages. TGFβ signaling leads to the phosphorylation of SMAD proteins and translocation of these mediators into the nucleus to control gene expression. Normal colonic epithelial cells displayed undetectable or low levels of phospho-SMAD2 (p-SMAD2) in approximately 50% of the cells (Fig. 4A–C). However, moderate to high levels of p-SMAD2, both in the cytoplasm and in the nucleus, were observed in primary metastatic colorectal carcinomas. Up to 100% (mean 77%) of the tumor cells showed strongly positive nuclear staining, which is indicative of active TGFβ signaling in the majority of primary CRC. These findings are in line with a previous report [22]. pSMAD2 staining in the primary CRC tumors was significantly higher than in normal colon (Fig. 4A–C). Surprisingly, many of the corresponding liver metastases had lost p-SMAD2 ($p < 0.0001$) (Fig. 4A–C). The presence of nuclear p-SMAD2 was not associated with increased vimentin expression (not shown).

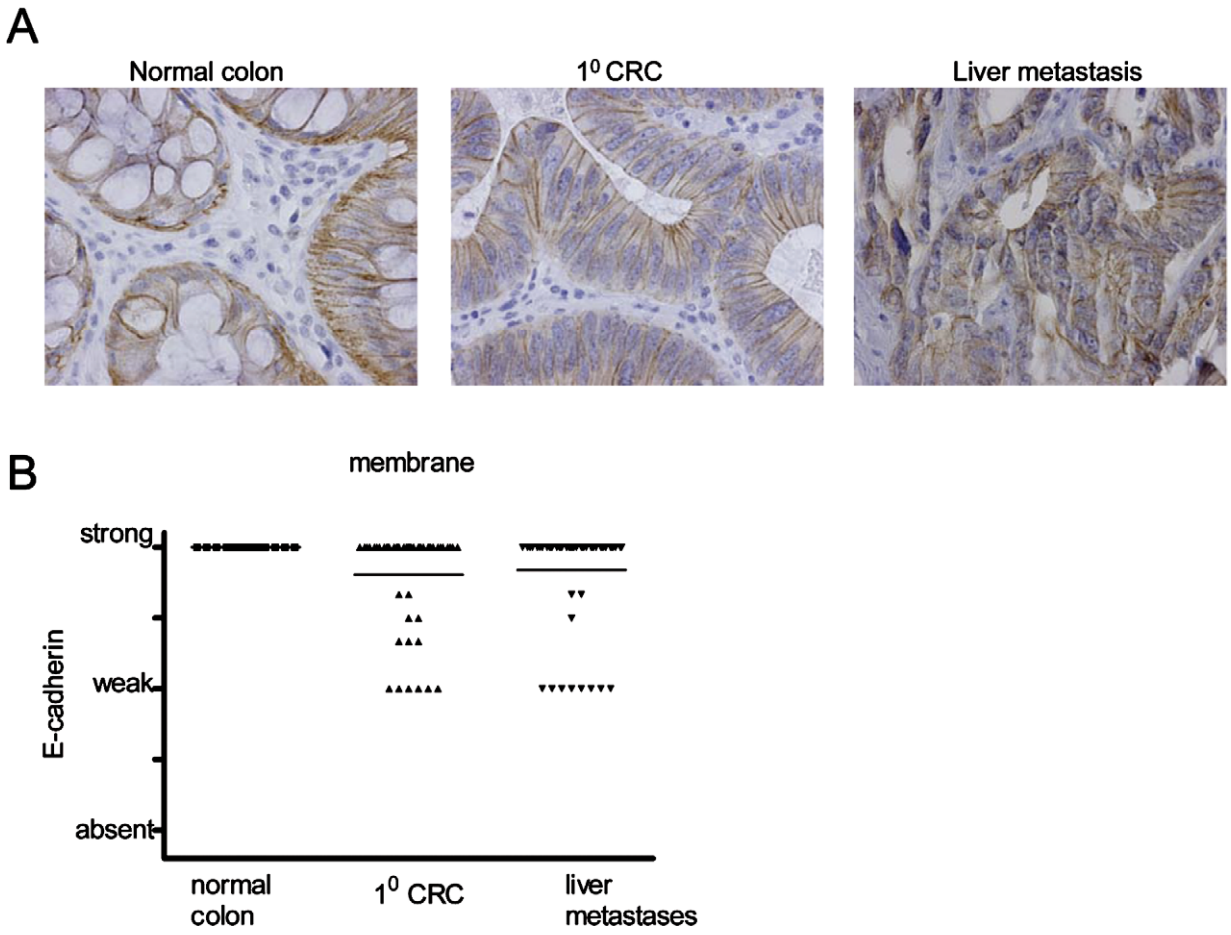


Fig. 2. Membrane localization of E-cadherin in primary CRC tumors and liver metastases. (A) Immunohistochemical analysis of E-cadherin expression and localization in normal colon epithelium, primary colorectal carcinoma and corresponding liver metastases. E-cadherin was expressed in all samples examined. (B) Scatter diagram demonstrating that a minority of primary tumors (13/57) and liver metastases (11/51) displayed a clear reduction in the amount of staining at cell–cell contacts.

### *3.6. Increased Notch signaling in primary colorectal tumors but not in metastases*

Previous work has shown that Notch signaling is activated in intestinal crypt cells and in intestinal adenomas in $APC^{min}$ mice [23]. To investigate Notch signaling in human colorectal cancer, we probed the TMAs for HES1, a well-established Notch target that acts as a transcriptional repressor. Normal colon tissue showed weak cytoplasmic HES1 staining but hardly any nuclear staining (2–3% of all cells) (Fig. 5). In contrast, the majority of primary metastatic colorectal tumors showed strong to very strong expression of HES1 in the cytoplasm and up to 50% of colorectal tumor cells showed strong nuclear HES1 staining (mean 24%). Thus, elevated Notch signaling may contribute to the development of colorectal carcinoma. To our knowledge this is the first report showing activated Notch signaling in human colorectal cancer. Interestingly, many metastases in the regional lymph nodes, the liver, the lungs and the brain showed a strongly reduced expression of HES1 when compared to their paired primary tumors ($p < 0.001$, Fig. 5).

## 4. Discussion

Our study shows that the activity of the Notch and TGF$\beta$ signaling pathways which regulate epithelial homeostasis in the gut and have been implicated in colorectal cancer development are differentially active in primary colorectal tumors and their corresponding metastases. Possibly, the activity of these pathways in primary and secondary colorectal tumors may depend

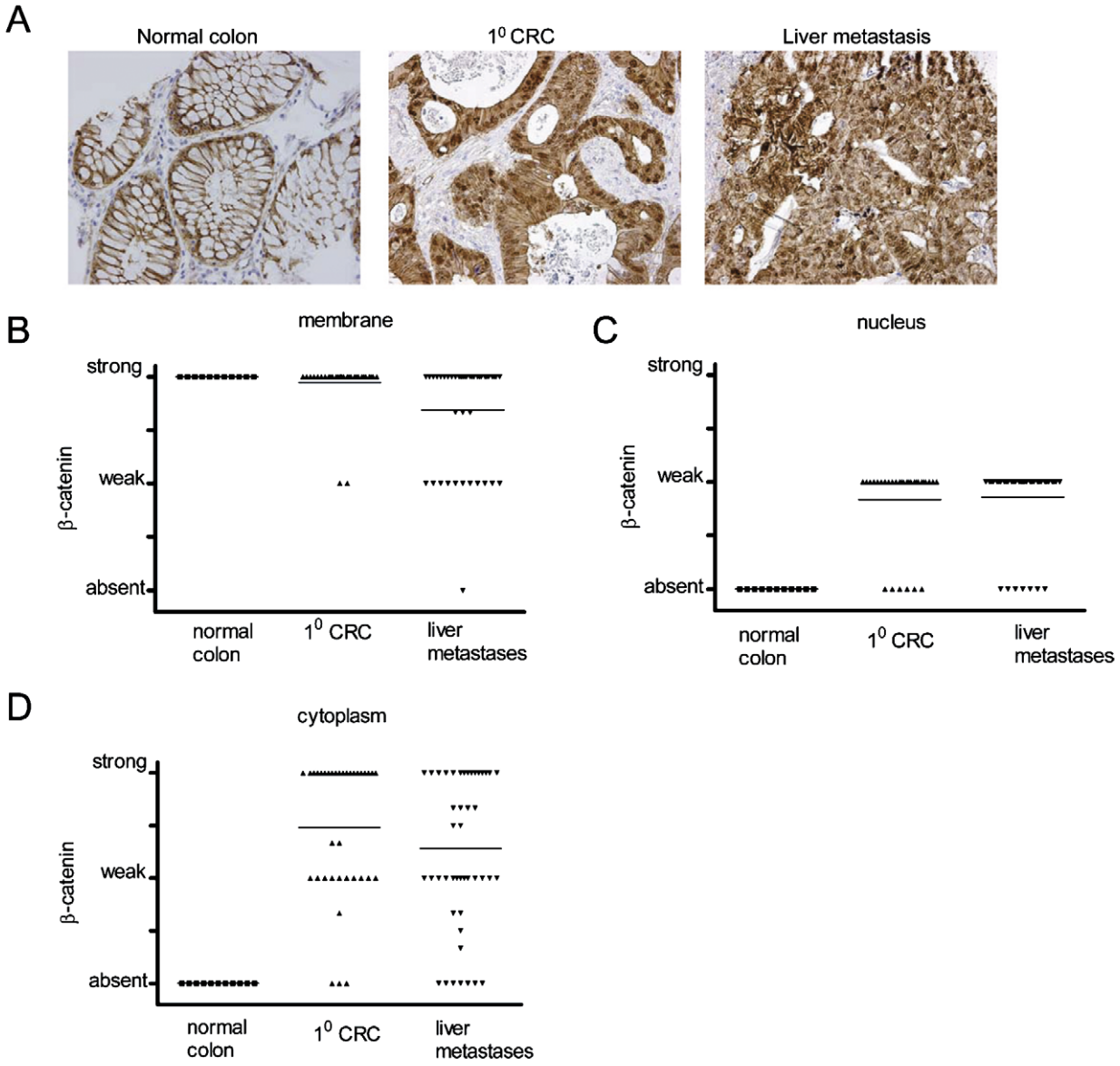


Fig. 3. Wnt pathway activation in primary CRC and liver metastases. (A) Immunohistochemical analysis of b-catenin expression and localization in normal colon epithelium, primary colorectal carcinoma and corresponding liver metastases. (B) Scatter diagram showing that the localization of $\beta$-catenin to cell–cell contacts is markedly lower in a minority of primary CRC tumors (2/51) and liver metastases (15/57) when compared to that in normal colon. (C) Nuclear $\beta$-catenin was observed in the majority of primary CRC tumors (45/51) and liver metastases (50/57). In normal colon tissue $\beta$-catenin was absent from the nucleus, as expected. (D) Scatter diagram demonstrating the elevated expression of $\beta$-catenin in the cytosol of primary CRC and liver metastases.

on the local production of the ligands that activate these pathways. Alternatively, genetic differences between primary colorectal tumors and their corresponding metastases [24–29] may underlie the differential activation of the Notch and TGF$\beta$ signaling pathways in primary and secondary tumors. Further work is required to distinguish between these possibilities.

Vimentin, a type III intermediate filament protein, is normally only expressed in cells of mesenchymal origin. Thus, our finding that vimentin is expressed in primary colorectal tumors suggests at least some degree of epithelial de-differentiation to a more mesenchymal phenotype. Vimentin expression in carcinomas is associated with local invasion and metastasis formation. We found that vimentin was moderately expressed in our panels of metastasizing and non-metastasizing

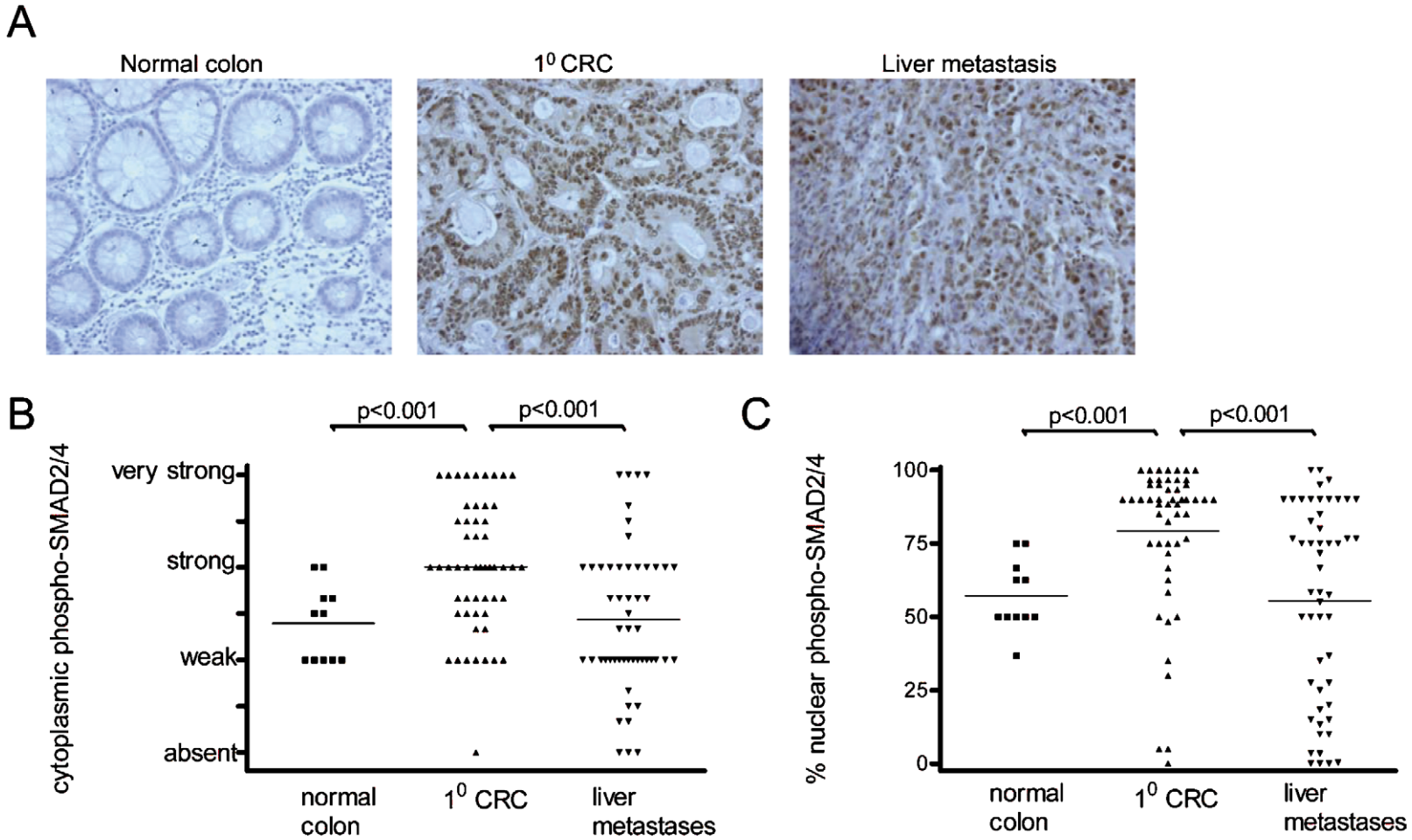


Fig. 4. TGF$\beta$ pathway activation in primary CRC. (A) Immunohistochemical analysis of phospho-SMAD2 on TMAs of normal colon epithelium, primary colorectal carcinoma and corresponding colorectal liver metastases. Scatter diagrams demonstrating increased expression of phospho-SMAD2 in the cytoplasm (B) and in the nucleus (C) of primary colorectal carcinomas, when compared to normal mucosa ($p < 0.001$). Many of the corresponding colorectal liver metastases show reduced levels of phospho-SMAD2 in the cytoplasm as well as in the nucleus ($p < 0.001$).

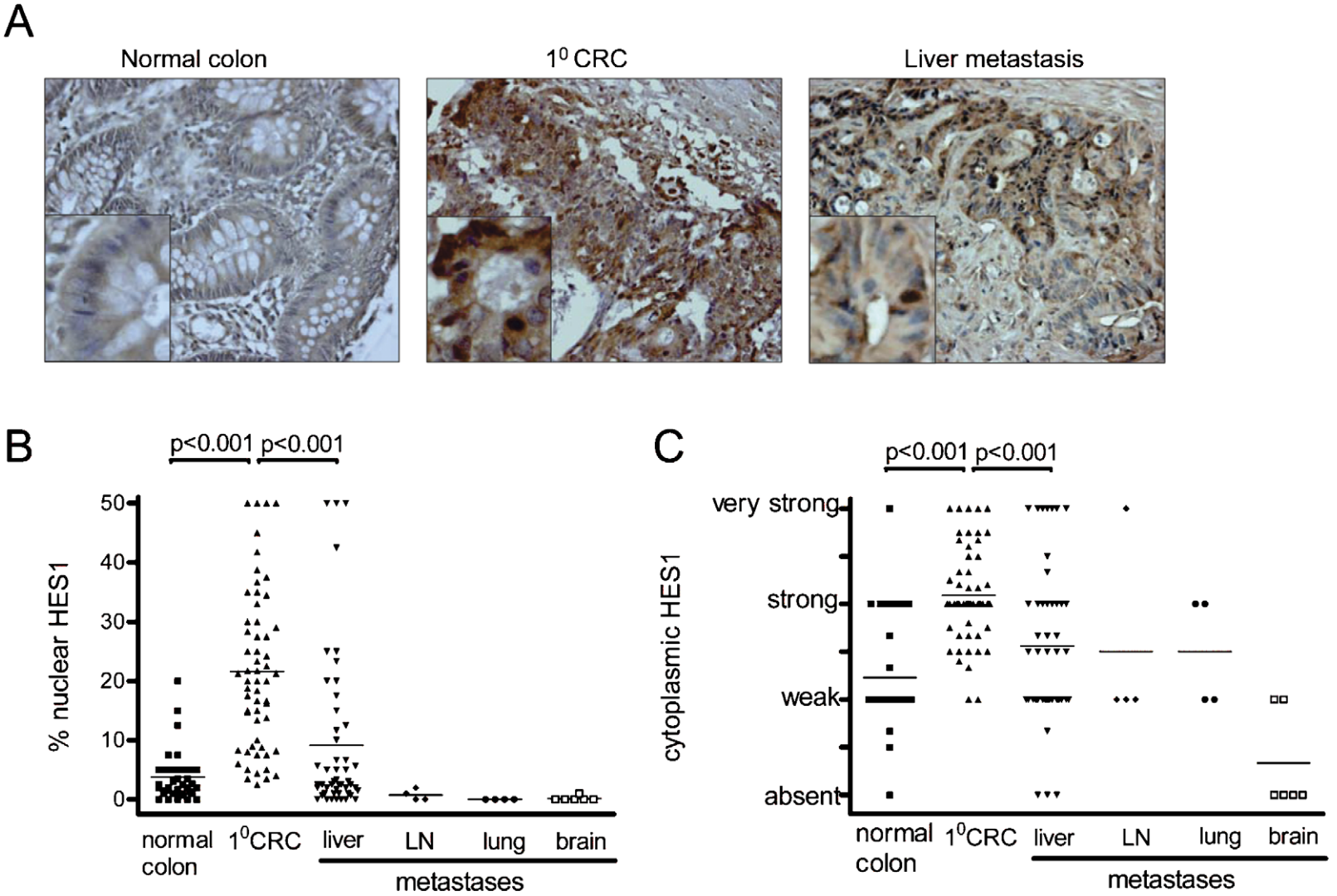


Fig. 5. Notch pathway activation in primary CRC. (A) Immunohistochemical analysis of the Notch target HES1 on TMAs of normal colon epithelium, primary colorectal carcinoma and corresponding colorectal liver metastases. (B, C) Normal colon showed weak cytoplasmic staining in the majority of cells, but only 3% of the cells displayed positive nuclei. Primary colorectal carcinomas showed strong HES1 staining in the cytoplasm and up to 50% of the tumor cells displayed high HES1 levels in the nucleus. The intensity of HES1 staining in the cytosol and in the nucleus of the corresponding metastases in the liver, lymph nodes (LN), lungs and brain was markedly lower than that in the primary tumors.

primary colorectal carcinoma samples. Thus, primary colorectal carcinomas are characterized by a general and moderate upregulation of vimentin which is not directly associated with metastatic potential (as both metastatic and non-metastatic primary tumors expressed vimentin). Vimentin expression was associated with mutations in the KRAS oncogene and with nuclear $\beta$-catenin. Ectopic expression of oncogenic H-Ras$^{V12}$, in concert with TGFb signaling, efficiently establishes a stable mesenchymal phenotype in epithelial cells and this is accompanied by the expression of vimentin [30]. Our results show that endogenous mutant KRAS and nuclear $\beta$-catenin, but not p-SMAD2 is associated with the expression of vimentin in human colorectal tumors. Despite the expression of this mesenchymal marker, the tumors retained their epithelial morphology.

The trans-differentiation of epithelial tumor cells is a process that shares some features with the epithelial-mesenchymal transitions (EMT) that occur during embryogenesis. The mechanistic insights into the pathways that regulate the EMT-like process in cancer cells is mostly derived from in vitro cell culture models [31]. While metastasis formation clearly requires tumor cells to dislodge from the primary tumor mass, invade the surrounding tissue, and gain access to the vascular or lymphatic systems, it is far less clear whether they have to undergo EMT-like changes to do this [32, 33]. Detached and invading tumor cells retain epithelial hallmarks like mucin production and cytokeratin expression. In addition, the morphological differentiation of distant metastases is often similar to that of the primary tumor. Also in the tumor series examined here we found no differences in morphological differentiation between primary colorectal tumors and their corresponding metastases (LMV PJvD, unpublished observations). Taken together, the concept that invading tumor cells undergo true trans-differentiation is disputable.

In addition to KRAS and TGF$\beta$ signaling, Notch signaling has also been implicated in the control of EMT [34,35]. Notch signaling is active in intestinal crypts and in adenomas in APC$^{min}$ mice [23] and Notch pathway inhibitors are attractive therapeutics in the treatment of intestinal neoplasia [36] by virtue of their ability to turn proliferative adenoma cells into goblet cells [23]. Our data show that Notch signaling is also strongly activated in primary human colorectal cancer. At present it remains unclear whether this is due to increased expression of Notch ligands, or to (epi)genetic changes in the tumor cells themselves.

Future work should establish whether metastases from colorectal cancer are dependent on Notch and TGF$\beta$ signaling pathways for their proliferation and survival.

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