NOTCH3 SIGNALING REGULATES MUSASHI-1 EXPRESSION IN METASTATIC COLORECTAL CANCER CELLS

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ABSTRACT

Musashi-1 (Msi-1) is a well-established stem cell marker in both normal and malignant colon cells and it acts by positively regulating the Notch pathway through inactivation of Numb, a Notch signalling repressor. To date, the mechanisms of regulation of Msi-1 levels remain largely unknown. Here we investigated the regulation of Msi-1 by Notch signalling in the colorectal cancer cell lines MICOL-14tum and LoVo and in primary cultures of colorectal cancer (CRC) metastases. Stimulation by the Notch ligand DLL4 was associated with an increase of Msi-1 mRNA and protein levels, and this phenomenon was prevented by the addition of antibody neutralizing Notch2/3 but not Notch1. Moreover, forced expression of activated Notch3 increased Msi-1 levels, whereas silencing of Notch3 by shRNA reduced Msi-1 levels in both CRC cells and tumor xenografts. Consistent with these findings, forced Notch3 expression or stimulation by DLL4 increased levels of activated Notch1 in MICOL-14tum and LoVo cells. Finally, treatment of CRC cells with anti-Notch2/3 antibody increased Numb protein while significantly reducing formation of spheroids in both MICOL-14tum cells and primary tumor cultures. This novel feed-forward circuit involving DLL4, Notch3, Msi-1, Numb and Notch1 may be relevant for regulation of Notch signalling in physiological processes as well as in tumor development. With regard to therapeutic implications, Notch3-specific drugs could represent a valuable strategy to limit Notch signaling in the context of colorectal cancers overexpressing this receptor.
INTRODUCTION

The Notch pathway is involved in intercellular communication and it regulates homeostasis of several tissues by controlling self-renewal, apoptosis, differentiation and proliferation of cells (1). In mammals, Notch signals through four different receptors (Notch1-4) and five different ligands (Jagged 1-2, Delta-like (DLL)-1,-3 and -4). Binding of a ligand triggers a proteolytic cascade that ultimately leads to the release of the intracellular domain (ICD) of the Notch receptor. ICD migrates to the nucleus, and interacts with the transcriptional complex CSL (C protein-binding factor 1/Suppressor of Hairless/Lag 1) converting it from a transcriptional repressor to a transcriptional activator; this eventually translates into increased transcription of target genes, including members of the Hes and Hey families (2). Increased Notch1 activity has been observed in various tumors (3), including intestinal tumors, where it is partially accomplished by β-catenin-mediated up-regulation of the Notch ligand Jagged-1 (4).

Aside from ligands, Notch activity is positively regulated by Musashi-1 (Msi-1), a RNA-binding protein of 39 kDa belonging to a conserved family of neural RNA-binding proteins composed of two RNA recognition motifs. Msi-1 binds the 3'-untranslated region of Numb, thus repressing its translation (5). Numb is a membrane-associated negative regulator of Notch signaling, which induces endosome-mediated degradation of Notch ICD, thus preventing its transmigration to the nucleus and eventual signaling (6). Numb involvement in asymmetric cell division is well-established: amounts of Numb and levels of Notch signaling are heterogeneous in daughter cells and this tunes cell differentiation versus stem cell maintenance (7).

Msi-1 was initially recognized as a RNA-binding protein required for asymmetric cell division of sensory organ precursor cells in Drosophila (8). Msi-1 was also proposed to be required for asymmetric distribution of intrinsic determinants in the development of mammalian nervous system; indeed, its expression is enriched in undifferentiated neuronal precursors or neuronal stem cells (9). Moreover, Msi-1+ cells were found in the mouse small intestine (10) and in the human...
colon at the base of the crypt compartment, where stem cells are considered to reside (11, 12), thus indicating that Msi-1 could label colon stem cells. Remarkably, despite its relevance for key physiologic processes, the mechanisms of regulation of Msi-1 levels remain largely unknown.

We recently demonstrated that Notch3 levels are significantly up-regulated in primary and metastatic colorectal cancer (CRC) samples compared to normal mucosa (13). Moreover, we also observed that a highly tumorigenic phenotype of CRC cells in xenograft models was associated with increased expression of various components of the Notch pathway. Forced expression of the active form of Notch3 mirrored the effects of DLL4 stimulation and promoted tumor formation (13), supporting an oncogenic role for Notch3 in CRC cells. In addition, recent evidence from the literature demonstrated up-regulation of Msi-1 expression in both primary tumors and metastatic CRC samples, compared to normal colon tissue (14).

Stimulated by the above observations, we sought to investigate whether Notch signaling could regulate Msi-1 levels in tumor cells. We found that Msi-1 expression is induced by DLL4 stimulation through a mechanism involving Notch3. In turn, increased Msi-1 levels sustain Notch1 signaling by repressing Numb. These observations highlight a novel feed-forward loop of regulation of Notch activity in cancer cells.
MATERIALS AND METHODS

Cell lines, primary samples and in vitro culture

Human liver metastasis samples were obtained from 25 patients bearing colon or rectal cancer following informed consent. Immediately after resection, tissues were washed in cold phosphate-buffered saline (PBS) containing Penicillin/Streptomycin (500 U/ml), gentamicin (1 µl/ml) and amphotericin (1.25 µg/ml). Samples were processed as described elsewhere (12). Briefly, the tissue was minced and incubated for 3 h at 37°C with collagenase (1.5 mg/ml) and hyaluronidase (20 µg/ml; both from Sigma-Aldrich, Milano, Italy) in DMEM/F12 medium (Gibco, Invitrogen, Carlsbad, CA). The digested material was centrifuged and sequentially filtered through 70 µm mesh; red blood cell lysis was performed at 37°C for 7 min in NH4Cl/KHCO3/EDTA buffer, and cell viability assessed by Trypan Blue dye exclusion. Before experimental use, cells were resuspended in DMEM/F12 medium, and maintained at 37°C in a 5% CO2 humidified atmosphere in low-adhesion plates for 1 week.

LoVo is a colorectal cancer cell line derived from a metastatic nodule from the supraclavicular region (15), whereas MICOL-14tum is a tumorigenic variant of MICOL-14, a cell line derived from a lymph node metastasis of rectal cancer (16). The MICOL-14tum cell line was established from a tumor developed in non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice after subcutaneous (s.c.) injection of parental MICOL-14 cells in Matrigel plus angiogenic factors (17). The cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS; Gibco, Invitrogen) and 1% L-glutamine, and used within 6 months from thawing and resuscitation.

To stimulate Notch signaling, P12 wells were coated with soluble recombinant human DLL4 (hDLL4, 4 µg/ml; R&D, Minneapolis, MN) in PBS/0.1% Bovine Serum Albumine (BSA, Sigma-Aldrich). One day later, MICOL-14tum, LoVo cells or primary cultures from tumor samples were added at a density of 2x10^5 cells/well in RPMI 1640 medium supplemented with 10% FCS and 1% L-glutamine.
L-glutamine (Invitrogen) and cultured for 72 h prior to analysis. The monoclonal antibodies (mAb), including anti-Notch1 (OMP-52M51), anti-Notch2/3 (OMP-59R5) and the control antibody (OMP-1B711), developed by OncoMed Pharmaceuticals Inc. (Redwood City, CA) in collaboration with MorphoSys AG (Martinsried, Germany) (18) were used at 10 μg/ml final concentration.

**Flow Cytometry (FACS) Analysis**

After 72 h of treatment, the cells were collected and incubated with Live/Dead dye (Invitrogen) for 30 min at 4°C, to stain dead cells. After washing in PBS, cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Non-specific binding was prevented by saturation with PBS/5% BSA, followed by incubation with rabbit anti-human Msi-1 (1:200; Abcam, London, U.K.) or mouse anti-human Muc-1 (1:100; Abcam) mAb. Cells were then stained with the appropriate secondary antibodies (Alexa 1:500; Invitrogen) and analyzed by FACS (LSR II, BD Biosciences, Franklin Lakes, NJ). Aldehyde dehydrogenase (ALDH) enzyme activity in viable cells was determined by a fluorogenic dye (Aldefluor® assay, Stem Cell Technologies, Grenoble, France) according to the manufacturer’s instructions. Briefly, 1×10⁶/ml cells were suspended in Aldefluor® assay buffer containing ALDH substrate (Bodipy-Aminoacetaldehyde) and incubated for 45 min at 37°C. As a control, cells were suspended in buffer containing Aldefluor® substrate in the presence of diethylaminobenzaldehyde (DEAB), a specific ALDH enzyme inhibitor. Data were collected from at least 5x10⁴ cells/sample on live cell gate, and analyses performed with Flow Jo (TreeStar, Ashland, OR).

**RNA extraction, reverse transcription and quantitative PCR (qRT-PCR) analysis**

Total RNA was extracted from cell populations by TRIzol (Invitrogen) according to manufacturer’s instructions. cDNA was synthesized from 0.5-1 μg of total RNA using the Superscript II reverse transcriptase (Invitrogen). Fifty-five ng of cDNA were used as a template.
and mixed with 10 μl of 2X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and primers (Suppl. Table 1) in a reaction volume of 20 μl. Cycling conditions were 10 min at 95°C, 60 cycles of 15 sec at 95°C and 1 min at 60°C. Each sample was run in duplicate on ABI PRISM® 7900HT Sequence Detection System (PE Biosystems, Foster City, CA). Results were analyzed using the comparative ΔΔCt method: data are presented as the fold difference in gene expression normalized to the housekeeping gene β2-microglobulin and relative to a relevant reference sample. qRT-PCR efficiency was in the range 95-105%.

**Western blotting**

After 72 h of treatment, the cells were harvested, lysed and subjected to SDS-polyacrylamide gel electrophoresis and Western blotting (WB). The membrane was saturated with PBS 5% non-fat dry milk (Sigma-Aldrich) for 1 h at room temperature. Immunoreactivity was evaluated by hybridization using the following antibodies: rabbit anti-Msi-1 (1:1,000 final dilution; Abcam), rabbit anti-Numb (1:500 final dilution; Millipore, Billerica, MA), rabbit anti-Notch1 ICD (1:1,000, final dilution; Val 1744, Cell Signaling, Danvers, MA) and mouse anti-α tubulin mAb (1:5,000 final dilution; Sigma Aldrich). The blots were then hybridized with a 1:5,000 dilution of HRP-conjugated anti-mouse or anti-rabbit antibody (Amersham-Pharmacia, Little Chalfont, U.K.). Finally, chemiluminescence was detected by SuperSignal kit (Pierce, Rockford, IL).

**Transduction of cells with viral vectors**

Lentiviral vectors encoding short harping RNA (shRNA) targeting human Notch3 (sh235, sh238) or a scrambled shRNA as a control were purchased from Sigma-Aldrich and used as previously reported (13). The N3ΔE retroviral vector, encoding constitutively active forms of human Notch3 and the control MX vector have been described elsewhere (19). The Notch2-ICD-encoding retroviral vector pMSVpuroICN2 was a kind gift of Adolfo Ferrando (Columbia University, NY). The human DLL4-EGFP and the control retroviral construct lacking DLL4 have been previously
described (20). Vector stocks were generated by a transient three-plasmid vector packaging system, as previously described (21). For transduction of MICOL-14\textsuperscript{tum} and LoVo cells, 200 μl of concentrated vector-containing supernatant were layered over target cells, previously seeded into 12-well culture plates at 1×10\textsuperscript{5} cells/well. After 6-9 h at 37°C, the supernatant was replaced with 2 ml complete medium. Assessment of transgene expression was performed 72-96 h after transduction.

**In vivo experiments and optical imaging of tumor**

NOD/SCID mice were purchased from Charles River (Wilmington, MA). Procedures involving animals and their care were performed according to institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L358, 12 December 1987). Implantation and expansion of CRC358 and CRC149 patient-derived tumor xenografts were performed as previously described (22, 23). Established tumors (average volume 500 mm\textsuperscript{3}) were treated with a control mAb or with the anti-Notch 2/3 mAb (40 mg/kg) by i.p. injection every two weeks. Tumor size was evaluated once-weekly by caliper measurements and the approximate volume of the mass was calculated using the formula \( V = \frac{4}{3}\pi \left(\frac{d}{2}\right)^{2} \frac{D}{2} \), where \( d \) is the minor tumor axis and \( D \) is the major tumor axis. When tumor volume reached 2500 mm\textsuperscript{3}, mice were sacrificed by cervical dislocation; tumors were harvested by dissection and subjected to flow cytometry analysis, or fixed in formalin and embedded in paraffin (FFPE) for histologic analysis.

To establish MICOL-14\textsuperscript{tum} xenografts, 5×10\textsuperscript{5} tumor cells were resuspended in PBS and s.c. injected in dorsolateral flanks of NOD/SCID mice, as previously reported (13). Prior to injection, MICOL-14\textsuperscript{tum} cells were transduced by a lentiviral vector encoding the luciferase gene. Anti-Notch2/3 mAb was administered by i.p. injection (40 mg/kg) the day following tumor cell injections and every 2 weeks thereafter. Bioluminescence signals were acquired on IVIS Imaging System (Xenogen Corporation, Alameda, CA, USA) as described elsewhere (24).
Histology and immuno-histochemical analysis

Quantification of necrosis was carried out after Hematoxylin/Eosin staining by a light microscope equipped with digital camera and MODEL software (Leica Microsystems). Immuno-histochemistry (IHC) was performed on FFPE samples using a standard avidin-biotin immunoperoxidase complex technique (Dako, Milano, Italy). Four-μm-thick sections were mounted on silanized slides, dewaxed in xylene, dehydrated in ethanol, boiled in 0.01M citrate buffer (pH 6.0) for 15 min in a microwave oven (95 °C), and incubated with 3% hydrogen peroxide for 5 min. After washing with PBS, samples were incubated in PBS/10% BSA for 30 min, followed by overnight incubation at 4°C with rabbit anti Msi-1 antibody (1:100; Abcam). Sections were subsequently incubated with biotinylated goat anti-rabbit immunoglobulin (Vectastain ABC kit, Vector Lab., Burlingame, CA) and peroxidase-conjugated avidin (Dako). Lastly, 0.02% diaminobenzidine and 1% hydrogen peroxide (Dako) in PBS were used as substrates in the color development reaction. Sections were then counterstained with hematoxylin. Immunoreactivity was scored for both the intensity and the percentage of marker-positive cells: intensity was given scores 0 to 3 (no staining = 0, light staining = 1, moderate staining = 2, and strong staining = 3) and percentage was given scores 1 to 6 (0–4% = 1, 5–20% = 2, 21–40% = 3, 41–60% = 4, 61–80% = 5, and 81–100% = 6). Finally, the two scores were multiplied to obtain the final score ranging from 0 to 18.

Immunofluorescence analysis

MICOL-14tum cells were plated on poly-D-lysine-coated glasses at 5x10⁴ cells/spot, fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Non-specific binding was prevented by incubation in PBS 5% BSA for 1 h. Staining was carried out by incubating cells with anti-Msi-1 (1:100; Abcam) mAb for 90 min, anti-CK18 (1:200; Abcam) mAb and anti-Notch1 ICD (1:800; Abcam) mAb for 30 min at room temperature. After three washes in PBS, appropriate secondary Alexa-conjugated antibodies (1:500; Molecular Probes, Invitrogen) were added for 1 h. Nuclear staining was performed by Topro-3 incubation (1:1,000; Invitrogen) for 10 min. The cells were
finally washed twice with PBS and mounted in glycerol. Confocal laser scanning microscopy was carried out with a Zeiss LSM 510 microscope (Zeiss, Jena, Germany) using Argon (488 nm) and Helium-Neon (543-633 nm) laser sources. Stained cells were analyzed by laser scanning microscopy. Data from 5 different fields for each section were used to calculate mean fluorescence intensity.

**Statistical Analysis**

Data of replicate experiments were shown as mean values ± Standard Deviation (SD). Comparisons between groups were made by the Student’s $t$-test.

Further experimental details are available in the Supplementary Data.
RESULTS

**hDLL4 up-regulates Msi-1 transcript levels in CRC cells by a Notch2/3-mediated mechanism**

We preliminary established conditions for ligand-mediated stimulation of Notch signaling in CRC cells. To this end, MICOL-14\textsuperscript{tum} cells transfected with the CSL-luc plasmid were stimulated by hDLL4 and Notch activity measured by \textit{in vitro} luciferase reporter assay 72 h later. An 8-fold increase in luciferase activity was observed in hDLL4-stimulated cells, compared to basal levels (Suppl. Figure 1A). Importantly, these effects were blocked by anti-Notch2/3 but not by anti-Notch1 mAb (Suppl. Figure 1A). Since MICOL-14\textsuperscript{tum} cells express Notch1-3 but lack Notch4 receptors (13), we could conclude that DLL4 stimulation triggers Notch signaling mainly through Notch2 and/or Notch3. These data were also supported by measurement of endogenous components of the Notch pathway by qRT-PCR. Indeed, in 5 independent experiments, stimulation of MICOL-14\textsuperscript{tum} cells with hDLL4 increased Notch3 and Hey-2 transcript levels by 5-8-fold, and this effect again was blocked by anti-Notch2/3 mAb (Suppl. Figure 1B). Similar effects were observed in primary cultures derived from CRC metastases (n=4); as shown in Suppl. Figure 1C, Notch-3 and Hey-2 transcript levels were significantly increased after hDLL4 stimulation and markedly decreased in the presence of anti-Notch2/3. At variance with MICOL-14\textsuperscript{tum} cells, incubation of primary tumor cells with anti-Notch2/3 significantly reduced levels of all Notch-related transcripts tested (including Notch1-3, Hey-1 and Hey-2) also in the absence of hDLL4 stimulation. Moreover, in primary samples hDLL4 increased levels of all components of the Notch pathway tested (including Notch2, Notch3, Hes-1 and Hey-2), but not Notch1 (Suppl. Figure 1C).

Next, we investigated the effects of DLL4 stimulation on the expression of stem cell-associated and differentiation markers. We observed that recombinant hDLL4 induced a 2-3-fold increase in Msi-1 levels in MICOL-14\textsuperscript{tum} cells, which was largely blocked by anti-Notch2/3 treatment (Figure 1A). We also observed increased levels of Mucin-1 (Muc-1) and Mucin-2 (Muc-2) mRNA following Notch2/3 blockade in cultures analyzed (Figure 1A). Since Muc-1 is a marker of...
secretory goblet cells (25), this finding supports previous observations indicating that Notch2/3 prevents colon progenitors from differentiating into secretory cells (26). In contrast, only marginal changes in Numb transcripts were detected in these experiments either in the presence or in the absence of hDLL4 (Figure 1A). To extend these findings, we stimulated LoVo cells with hDLL4. In this case, however, only Msi-1 mRNA levels increased significantly, whereas no changes were detected in Muc-1, Muc-2 or Numb levels (Figure 1B). Moreover, treatment with anti-Notch 2/3 blocked the effects of hDLL4 on Msi-1 levels, and increased Muc-1 transcript levels (Figure 1B). Finally, hDLL4 led to increased Msi-1 expression and to a corresponding decrease in Muc-1 levels also in primary cultures derived from liver CRC metastases, whereas only marginal changes were detected in Muc-2 and Numb levels, and these modulations were completely blocked by anti-Notch2/3 (Figure 1C). Of note, treatment of primary tumor cultures with anti-Notch2/3 was associated with a significant reduction of Msi-1 expression and concomitant increase of Muc-1 and Muc-2 levels also in the absence of hDLL4 stimulation (Figure 1C), probably reflecting higher endogenous levels of Notch signaling, compared to MICOL-14<sup> tum </sup>cells.

Up-regulation of Msi-1 levels was also observed following retroviral vector-mediated delivery of hDLL4 cDNA in MICOL-14<sup> tum </sup>cells (Figure 1D), thus confirming results obtained with recombinant hDLL4. To test whether the effect of hDLL4 was restricted to Msi-1 expression, we addressed mRNA levels of several stem cell-associated genes in MICOL-14<sup> tum </sup>and LoVo cells after hDLL4 stimulation. As reported in Figure 1E, DLL4 stimulation significantly increased Msi-1 levels and a trend towards increased ALDH1A expression was detected, whereas Bmi-1, EpCAM and EphB2 mRNA levels did not substantially change. Since it is reported that Notch signaling up-regulates ALDH (27), we evaluated by flow cytometry ALDH activity in both MICOL-14<sup> tum </sup>and LoVo cells. As reported in Figure 1F, hDLL4 stimulation was followed by a significant increase in ALDH activity in both tumor cell lines.

**Effect of Notch2/3 blockade on Msi-1 and Numb protein levels in CRC cells**
To corroborate these findings, we investigated Msi-1 expression at protein level in primary cultures of 5 liver CRC metastases by flow cytometry. As shown in Figure 2 (panel A and B), increased numbers of Msi-1+ cells were found following hDLL4 stimulation; moreover, anti-Notch2/3 mAb strongly reduced the percentage of Msi-1+ cells in both hDLL4-stimulated and control cell cultures (Figure 2A-B), whereas treatment with anti-Notch1 only had marginal effects on Msi-1 expression (Figure 2A). Confocal immunofluorescence analysis confirmed that hDLL4 stimulation increased Msi-1 expression in MICOL-14 tum cells, and this effect was blocked by anti-Notch2/3 mAb (Figure 2C). In contrast, the expression of CK18, a common epithelial marker for colon cancer cells, was not affected by these treatments (Figure 2C). Notably, anti-Notch2/3 treatment markedly reduced Msi-1 protein levels also in the absence of hDLL4 stimulation (Figure 2A-C), exceeding variations in Msi-1 transcript levels seen under the same experimental conditions (Figure 1B). Finally, we analyzed reciprocal effects of hDLL4 stimulation and anti-Notch2/3 blockade on Numb protein levels by Western blotting. As shown in Figure 2D, hDLL4 stimulation was associated with a small decrease in Numb protein levels, whereas anti-Notch2/3 mAb clearly increased Numb protein in both control and hDLL4-stimulated MICOL-14 tum and LoVo cells, as well as in primary tumor cell cultures (Figure 2D). Notably, variations in Numb protein levels were accompanied by minor changes in Numb transcript levels (Figure 1), in line with the idea that Numb protein modulation could be accounted for by Msi-1, which mainly affects post-transcriptional mRNA processing (5).

**Msi-1 modulation is Notch-3 dependent**

To clarify which Notch paralog mainly controls Msi-1 expression, we overexpressed either Notch3 (N3) or Notch2 (N2) ICD in MICOL-14 tum cells by using retroviral vectors. Forced N3ICD expression increased Msi-1 levels by 3-fold, together with up-modulation of Hey-2 levels (Figure 3A). In contrast, over-expression of N2ICD increased Hey-2 but caused minimal changes in Msi-1 mRNA levels (Figure 3A). These results were confirmed in LoVo cells, where forced N3ICD...
expression strongly increased Msi-1, Notch3 and Hey-2 levels (Figure 3B). Altogether, these results fit the hypothesis that hDLL4 could up-regulate Msi-1 by a Notch3-mediated mechanism. As a complementary approach, Msi-1 expression was investigated in MICOL-14\textsuperscript{tum} cells transduced by lentiviral vectors encoding Notch3-specific shRNA (sh235 and sh238). In agreement with our previous findings (13), Notch3 silencing resulted in 40% to 60% decrease in Notch3 mRNA levels (Figure 3C), compared to control shRNA cells; in addition, significantly lower levels of Msi-1 and Hey-2 were recorded upon Notch3 silencing, compared to control cells (Figure 3C). In line with these \textit{in vitro} findings, IHC analysis demonstrated very low Msi-1 expression in xenotransplanted tumors generated by MICOL-14\textsuperscript{tum} cells with attenuated Notch3 levels (Figure 3D). Moreover, treatment of MICOL-14\textsuperscript{tum} xenografts with anti-Notch2/3 mAb caused a small but significant reduction of tumor burden measured by optical imaging (Figure 4A), associated with marked reduction of Msi-1 expression, increased Muc-1 levels (Figure 4B) and reduction of cell proliferation, measured by Ki67 staining (Figure 4C).

To strengthen these findings, we decided to further investigate the \textit{in vivo} consequences of Notch3 inhibition on Msi-1 levels in patient-derived tumor xenografts. First, we mined gene expression data from a large collection of patient-derived xenografts of metastatic colorectal carcinomas (22) and identified two cases bearing high-level expression of Notch3 transcript (CRC 358 and CRC 149). Notch3 overexpression was confirmed at the protein level (data not shown). Then, we treated mice bearing large CRC 358 and CRC 149 tumor xenografts (average size 500 mm\textsuperscript{3}) with the anti-Notch2/3 or a control mAb. Analysis of tumor samples by flow cytometry disclosed a strong reduction in Msi-1 levels compared to control tumors, and a concomitant increase in Muc-1 expression (Figure 4D-E). All the above findings indicate that attenuation of Notch3 signaling by shRNA or mAb administration decreases Msi-1 expression in tumor cells, both \textit{in vitro} and \textit{in vivo}.

\textbf{Effects of Notch2/3 blockade on the ability to form spheroids}
Msi-1 is a well-recognized colon stem cell marker (11, 12) and one of the canonical features of cancer stem cells is their ability to form spheroids when cultured under specific in vitro culture conditions. We thus wondered whether experimental reduction of Msi-1 expression could affect the number of spheroid-forming cells. To test this hypothesis, we performed an ELDA assay with primary tumor cultures in the presence or in the absence of anti-Notch2/3 mAb. As shown in Figure 5A, the frequency of spheroid-forming cells was significantly reduced in the presence of the anti-Notch2/3 antibody from 1/738 to 1/239. Similar results were obtained for LoVo and MICOL-14 tum cells: the spheroid-forming cell frequency decreased significantly in the presence of anti-Notch2/3 antibody (Figure 5A). These results seemed to indicate that Notch2/3 blockade could at least in part affect cancer stem cell properties by modulating Msi-1 expression. In fact, frequency of spheroid-forming cells was significantly increased following over-expression of activated Notch3 in MICOL-14 tum cells (Figure 5B) whereas it decreased, albeit not attaining statistical significance (p=0.075), following attenuation of Notch3 levels by shRNA. On the whole, these results strongly indicated that Notch3 levels could impact on the clonogenic potential of CRC cells.

**Effect of Notch3 modulation on Notch1 protein**

Since Numb is an established negative regulator of Notch1 ICD downstream of Msi-1 [21] and Notch3 regulates Msi-1 expression, we argued that Notch3 could indirectly behave as a regulator of activated Notch1 levels. Indeed, stimulation with hDLL4 was followed by increased nuclear levels of activated Notch1 in MICOL-14 tum cells (Figure 6A), which are known to respond to hDLL4 stimulation mainly through Notch3 receptors (see Suppl. Fig. 1). Similar results were obtained following transfection of MICOL-14 tum cells with the N3ΔE (Figure 6B). These results were further supported by Western Blot analysis that disclosed a significant (p=0.002) increase in N1ICD protein in MICOL-14 tum and LoVo cells over-expressing N3ΔE compared to controls (Figure 5C, n=4 independent experiments). Although Notch3 is a recognized transcriptional target
of Notch1 in certain cell types, this is the first direct evidence that Notch3 is part of a feed-forward circuit which stabilizes activated Notch1 in tumor cells.
DISCUSSION

Although tumor cells often express multiple Notch paralogs, the reciprocal interplay between these receptors is not well understood. In T acute lymphoblastic leukemia (T-ALL), where activating mutations of Notch1 are common, Notch3 is considered as a canonical transcriptional target of Notch1 (28). Notably, Notch3 can also contribute to the oncogenic process in T-ALL, as shown by genetic studies in transgenic mice (29), although its transcriptional activity may be weaker compared to Notch1 due to the lack of the transactivation domain (30). In solid tumors, Notch3 signaling accounts for oncogenic features in breast (31), ovarian (32), lung (33) and colorectal cancer (13). However, as tumor samples often simultaneously co-express Notch1 receptors, it is difficult to address the real contribution of Notch3 to Notch signaling in tumor cells. In particular, whether Notch3 - in addition to its transcriptional activity on target genes shared with Notch1 - could influence Notch1 signaling by other routes has not been as yet investigated.

In this work, based on the initial finding of increased Msi-1 levels in CRC cells after DLL4 stimulation, we postulate a possible novel mechanism of regulation of Notch1 activity by a feed-forward circuit involving Notch3 and Msi-1 (Figure 7). Msi-1 is a RNA-binding protein that acts as a positive regulator of Notch activity by binding Numb mRNA and blocking its translation, thus inhibiting Numb-mediated degradation of Notch1 ICD. It is important to stress that Numb regulates Notch1, but not Notch3 poly-ubiquitination in mammalian cells (34). Based on these data, variations in Numb levels that we observed downstream of Notch3 are likely to impact on the accumulation of activated Notch1 but not Notch3 in tumor cells.

Despite its established role in neural stem cells and as a marker of intestinal and mammary stem cells (9, 35), the molecular cues responsible for Msi-1 up-regulation in cancer cells are largely unknown. In this study, by using complementary approaches (including loss-of-function experiments with neutralizing mAb or shRNA and gain-of-function experiments with retroviral vectors), we demonstrate for the first time that Notch3 affects Msi-1 levels in both tumor cell lines and primary tumor samples. In these latter, anti-Notch2/3 caused a significant attenuation of Msi-1
expression and a corresponding increase in Muc-1 levels also in the absence of DLL4 stimulation. This finding might reflect higher endogenous Notch activity in primary tumor cells compared to colon cancer cell lines, as previously observed by others (36). Although the experiments with anti-Notch2/3 mAb do not allow dissecting the contribution of these two receptors in the regulation of Msi-1 levels, the key role played by Notch3 is strongly supported by shRNA-mediated silencing of Notch3 in CRC cells, as well as by converse experiments (i.e. the experimental elevation of Notch3) and the lack of variations in Msi-1 levels following forced Notch2 ICD expression.

The mechanisms connecting Notch3 to Msi-1 remain as yet unknown. In any case, transfection of a plasmid bearing Msi-1 promoter sequences upstream of the luciferase gene (37) into MICOL-14tum cells overexpressing the activated Notch3 did not result in increased luciferase activity compared to control cells (Suppl. Figure 2). Moreover, bio-informatics analysis did not disclose classic CSL consensus binding sequences in the promoter region of Msi-1 (not shown), nor was the Msi-1 promoter found among genes bound by the active Notch3 complex in ChIP-on-Chip studies in ovarian cancer cells (38). Altogether, these results suggest that Notch3 could modulate Msi-1 levels by indirect mechanisms. In this regard, it was recently observed that several tumor suppressor microRNA (miR) act as direct regulators of Msi-1, affecting its expression pattern during tumorigenesis of brain tumors (39), opening the possibility that Notch3 controls Msi-1 expression by miR-mediated mechanisms. To test this hypothesis, future studies will address whether miR involved in regulation of Msi-1 are downstream on Notch3 in CRC cells.

Previous studies focusing on Numb have revealed a reciprocal negative regulation between Notch and Numb. In fact, it was shown that high levels of Notch signaling down-regulate Numb and Numb-like in mammalian cells (40). This phenomenon requires the PEST domain in the Numb-like protein and it is blocked by the proteasome inhibitor MG132, eventually implying that it could be due to increased protein turnover. In that study, however, the possible contribution of Notch3 and Msi-1 to modulation of Numb expression was not addressed. In our experiments, the reduction of Numb is likely accounted for by the effects of Msi-1 on Numb mRNA translation. These two
complementary mechanisms converge on Numb to keep its levels low under sustained Notch signaling. In addition to Numb, Msi-1 has been shown to repress translation of mRNA encoding for the cell cycle inhibitor p21\(^{waf}\) (41); therefore, in addition to Numb other Msi-1 targets could contribute to the biological effects observed.

These findings may be of relevance for physiological processes as well as for tumor development. In this respect, it is interesting to note that DLL4 stimulation was associated with markedly increased ALDH1A expression and ALDH activity. ALDH has been reported to be a specific marker for colon stem cells and it has been used to track stem cell populations during colon cancer development (42). These results confirm recent studies indicating that Notch signaling regulates ALDH activity both in normal stem cells and in cancer cells (27, 43, 44). With regard to therapeutic implications, whether anti-Notch3 therapy could be clinically effective by promoting differentiation of colorectal cancer cells possibly increasing their sensitivity to chemotherapy is an intriguing issue that deserves further investigation.

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REFERENCES


FIGURE LEGENDS

Figure 1. hDLL4-mediated increase of Msi-1 levels is counteracted by Notch2/3 blockade

A-C) Measurement by qRT-PCR of genes associated with stemness (Msi-1, Numb) or differentiation (Muc-1 and Muc-2) after 72 h stimulation with hDLL4 in MICOL-14tum cells (panel A), LoVo cells (panel B) or primary cultures from CRC liver metastasis (panel C) in the presence of anti-Notch2/3 or control mAb. Data are expressed as mean ± SD of five (panel A) and four (panel B and C) different experiments. *p<0.05.

D) qRT-PCR following forced expression of hDLL4 into MICOL-14tum cells. Msi-1, Notch3 as well as Hey-2 significantly increased whereas no substantial alteration in Numb RNA levels was detected compared to control (CTRL) or parental (WT) cells. Data are expressed as mean ± SD of three different replicates. *p<0.05.

E) qRT-PCR of additional stemness markers in MICOL-14tum and LoVo cells after 72 h of stimulation with hDLL4. Msi-1 is the only marker significantly increased by hDLL4; data are expressed as mean ± SD of three (LoVo) and four (MICOL-14tum) different experiments. *p<0.05.

F) Flow cytometry analysis of ALDH activity in MICOL-14tum and LoVo cells after stimulation with hDLL4. Gates are set on the isotype control and values indicate the percentage of ALDH+ cells. The histogram on the right represents the mean percentage ±SD of four experiments in MICOL-14tum and three experiments in LoVo cells.

Figure 2. Notch2/3-dependent increase of Msi-1 protein levels by hDLL4 in CRC cells

A) Analysis of Msi-1 expression in primary cultures from liver CRC metastases cultured for 72 h in hDLL4-coated wells (or BSA as a control) in the presence and in the absence of anti-Notch2/3, anti-Notch1, or control (CTRL) mAb. Data are expressed as mean ± SD of the percentage of Msi-1+ cells, according to flow cytometric analysis (n=3-5 experiments). *p<0.05.
B) Representative analysis of Msi-1 expression in a primary culture from a liver CRC metastasis. hDLL4 stimulation increased Msi-1 expression and this effect was completely blocked by anti-Notch2/3 mAb. Gates are set on the isotype control and values indicate the percentage of Msi-1+ cells.

C) On the left, immunofluorescence analysis of Msi-1 expression in MICOL-14 tum cells cultured for 72 h in the presence of hDLL4 or BSA, with anti-Notch2/3 or a control mAb. Nuclei were stained with TOPRO-3; the cytoplasm was stained with anti-CK18. Magnification 40x. The histogram on the right represents mean fluorescence intensity for Msi-1 (488 channel) calculated as described in Materials and Methods. *p<0.05

D) Western Blot analysis of Msi-1 and Numb expression in CRC cells. Protein lysates were obtained from MICOL-14 tum, LoVo cells and primary samples of CRC metastases cultured as described above; α-tubulin was used to normalize loading of the lanes. One representative experiment out of three is shown.

Figure 3. Notch3 contributes to regulation of Msi-1 levels in CRC cells and patient-derived tumor xenografts

A-B) qRT-PCR after forced expression of activated Notch3 (N3ΔE) or Notch2 (N2ICD) into MICOL-14 tum (panel A) or LoVo (panel B) cells. The columns show relative levels of Notch2, Notch3, Msi-1 and Hey-2 transcripts. Data are expressed as mean ± SD of three different replicates. *p<0.05.

C) qRT-PCR after shRNA-mediated Notch3 silencing (sh235, sh238) in MICOL-14 tum. Notch3, Hey-2 and Msi-1 levels were significantly decreased compared to control vector (shRNA) or parental (WT) cells. Data are expressed as mean ± SD of three different replicates. *p<0.05.

D) IHC analysis of Msi-1 expression in tumor xenografts formed by MICOL-14 tum cells bearing attenuated levels of Notch3 expression (sh235) or control cells (shRNA) in NOD/SCID mice. Two
different samples per group are shown at a 20x and 40x magnification. Columns show mean ±SD score of n=4 samples, calculated as detailed in Materials and Methods.

**Figure 4. Notch3 blockade affects Msi-1 and Muc-1 expression in CRC xenografts**

A) Optical imaging of s.c. tumors formed by MICOL-14\textsuperscript{tum} cells labeled with the luciferase gene. Left panel: representative images acquired at day 10 and 23 after tumor cell injection of three representative control (CTRL) or anti-Notch2/3-treated mice. Mice were treated with anti-Notch2/3 (OMP-59R5) or control antibody (OMP-1B711), both dosed at 40 mg/kg, at day 0 and day 14. Right panel: quantitative analysis of luciferase activity expressed as mean ± SD of 10 tumors/group showing significant reduction of tumor burden in anti-Notch2/3-treated mice. *p<0.05.

B) Modulation of Msi-1 and Muc-1 expression in tumor samples from anti-Notch2/3-treated or control mice by flow cytometry; columns indicate mean ± SD of the percentage of Msi-1\textsuperscript{+} or Muc-1\textsuperscript{+} cells (n=10 samples/group).*p<0.05.

C) Reduced Ki67 expression in tumor samples from anti-Notch2/3-treated compared to control mice by flow cytometry; columns indicate mean ± SD of the percentage of Ki67\textsuperscript{−} cells (n=10 samples/group).*p<0.05.

D) Validation of results in patient-derived xenografts. Msi-1 and Muc-1 expression was analyzed by flow cytometry in xenografts obtained following s.c. injection of CRC 358 or CRC 149 cells into NOD/SCID mice and treatment with anti-Notch2/3 or control mAb of established tumors as detailed in Materials and Methods. Columns indicate mean ± SD of the percentage of Msi-1\textsuperscript{+} or Muc-1\textsuperscript{+} cells (n=4 samples/group).*p<0.05.

E) Left panel, representative flow cytometry analysis of Msi-1 and Muc-1 expression in tumor samples. Filled peaks represent the isotype control and empty peaks indicate the percentage of Msi-1\textsuperscript{+} or Muc-1\textsuperscript{+} cells.
Figure 5. Effects of Notch3 blockade on spheroid-forming ability

A) ELDA of MICOL-14\textsuperscript{tum}, LoVo cells and primary cultures from CRC metastases. Cells were cultured at various concentrations for 4 days in the presence of anti-Notch2/3 or CTRL antibody. Data were analyzed by a specific web-tool (as detailed in Material and Methods) and expressed as mean values \(\pm\) SD; *\(p<0.05\). As shown in the box, spheroids obtained in the presence of anti-Notch 2/3 antibody were smaller than control spheroids, although the difference in spheroid diameter (~20\%) was not statistically significant.

B) ELDA of Notch3-overexpressing (N3\textDeltaE) or control (MX), sh235- or shRNA- transduced MICOL-14\textsuperscript{tum} cells. The number of spheroid-forming cells was evaluated after 4 days in PhEMA-treated plates and analyzed by a specific web-tool (as described in Material and Methods); data are expressed as mean values \(\pm\) SD. *\(p<0.05\).

Figure 6. Notch3 contributes to regulate Notch1 activity in CRC cells

A) Immunofluorescence analysis of MICOL-14\textsuperscript{tum} cells cultured for 72 h in hDLL4-coated wells reveals increased activated Notch1 levels (actN1) compared to control (BSA); nuclei were stained with TOPRO3. Magnification 20x. Columns show mean fluorescence intensity of actN1 (488 channel), calculated as detailed in Materials and Methods. *\(p<0.05\).

B) Immunofluorescence analysis of N1ICD expression in MICOL-14\textsuperscript{tum} cells transduced by a retroviral vector encoding activated Notch3 (N3\textDeltaE); nuclei were stained with TOPRO3. Magnification 40x. Columns show mean fluorescence intensity of actN1 (488 channel), calculated as detailed in Materials and Methods. *\(p<0.05\).

C) Western Blot analysis of N1ICD expression in lysates of MICOL-14\textsuperscript{tum} (left panel) and LoVo cells (right panel) transduced by a vector encoding activated Notch3 (N3\textDeltaE), an empty vector (MIX) or parental cells (WT). One representative Western blot is shown in the upper part of the panel. Columns report results of three independent experiments. *\(p<0.005\).
**Figure 7. A novel feed-forward circuit of regulation of Notch signaling in tumor cells**

Top panel: Triggering of the Notch3 receptor by DLL4 increases Msi-1 levels in CRC cells. This is followed by repression of Numb-mediated inhibitory effects and increased Notch1 signaling. The mechanism can reinforce direct activation of Notch1 by DLL4. Activated Notch1 on its own further sustains transcription of Notch3, thus reinforcing the circuit. Bottom panel: Inhibition of Notch3 (i.e. by neutralizing antibody) is one possibility of weakening Notch signaling by interfering with Msi-1-mediated effects.
Figure 4

A

CTRL

anti-Notch2/3

day 10 p.i.

day 33 p.i.

B

CTRL

anti-Notch2/3

Positive cells [%]

Msi-1

Muc-1

C

CTRL

anti-Notch2/3

Ki67 positive cells [%]

D

CTRL

anti-Notch2/3

Positive cells [%]

CRC358

CRC149

CRC358

CRC149

Msi-1

Muc-1

E

CTRL

anti-Notch2/3

CRC358

Ki67

Msi-1

Muc-1

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Figure 7
NOTCH3 SIGNALING REGULATES MUSASHI-1 EXPRESSION IN METASTATIC COLORECTAL CANCER CELLS

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