SUPPLEMENTARY DATA

MATERIALS AND METHODS

Reporter gene assay

MICOL-14^{tum} cells were transiently co-transfected using lipofectamine 2000 (Invitrogen) with CSL-luc, a Notch-responsive reporter plasmid carrying the luciferase reporter gene under the control of a promoter containing CSL binding sites (1), and a plasmid encoding β-galactosidase under the control of the human cytomegalovirus (CMV) promoter, used to normalize transfection efficiency. One day later, the cells were split and cultured for an additional 48 h in wells coated with hDLL4 as above, or BSA as a control. Each experiment was repeated at least three times. Cell lysates were harvested 72 h post-transfection, and luciferase and β-galactosidase assays were carried out using BriteLite Plus (Perkin-Elmer, Waltham, MA) and Tropix® Galacto-Light™ (Applied Biosystems, Invitrogen), respectively, on a plate luminometer (Perkin-Elmer, Waltham, MA).

To test possible direct effects of activated Notch3 on the Msi-1 promoter, in a set of experiments MICOL-14^{tum} cells were transfected with the pMsi1-7-Luc (100 ng per well) (2) and pRL-CMV (1 ng/well; Promega, Madison, WV) vectors using the Exgen transfection reagent (Euromedex, Paris, France). Luciferase activity was measured 48 h after transfection using the luciferase dual system (Promega).

Spheroid formation assay

To determine the frequency of spheroid-forming cells we performed an extreme limiting dilution analysis (ELDA) in the presence and in the absence of anti-Notch2/3 mAb (10 μg/ml). Briefly, primary tumor cells, as well as untreated and transduced MICOL-14^{tum} and LoVo cells, were plated at different concentrations in 96-well flat-bottom ultra-low attachment Phema-coated plates (BD Bioscience) in a total volume of 0.1 ml of DMEM/F12 medium for primary tumor cells and RPMI 1640 for MICOL-14^{tum} and LoVo cells; both media were serum-free and supplemented with

Epithelial growth factor (EGF, Peprotech, London, U.K.) and basic Fibroblast growth factor (bFGF, Peprotech). Twenty replicate wells were set up for each cell concentration. After 4-day incubation, the wells were scored for spheroid formation; the frequency of spheroid-forming precursor in each population was calculated by using the ELDA web tool as described elsewhere (3).

REFERENCES

1. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004;306:269-71.

2. Rezza A, Skah S, Roche C, Nadjar J, Samarut J, Plateroti M. The overexpression of the putative gut stem cell marker Musashi-1 induces tumorigenesis through Wnt and Notch activation. J Cell Sci 2010;123:3256-65.

3. Pastò A, Marchesi M, Diamantini A, Frasson C, Curtarello M, Lago C, et al. PKH26 staining defines distinct subsets of normal human colon epithelial cells at different maturation stages. PLoS ONE 2012:e43379.

SUPPLEMENTARY FIGURES

Figure S1. Notch2/3 neutralization impairs Notch signaling in CRC cells

A) MICOL-14^{tum} cells were lipofected with CSL-luciferase and β-galactosidase encoding plasmids and cultivated for 72h in wells coated with recombinant human DLL4 (hDLL4) or bovine serum albumine (BSA) in the presence of various antibodies. Luciferase activity was measured by a luciferase reporter assay and normalized to β-galactosidase activity. Data are expressed as mean ± SD of three independent experiments. *$p<0.05$.

B-C) Measurements by qRT-PCR of expression levels of components of the Notch pathway after 72 h of stimulation with hDLL4 in MICOL-14^{tum} cells (panel B, n=5 replicates) or primary cultures from CRC liver metastases (panel C, n=4 different samples). Data are expressed as mean ± SD. *$p<0.05$.

Figure S2. Study of the Msi-1 promoter activity *in vitro*

The pMsi1-7-luc reporter was transfected into MICOL-14^{tum}-MX (control) or MICOL-14^{tum}-N3ΔE (transduced with N3ICD) cell lines as indicated. Note that Msi-1 promoter activity appears similar in the two cell lines. The picture is representative of three independent experiments, each with four technical replicates. Histograms represent mean ± SD (n=4). ** $p<0.01$.

Figure S1

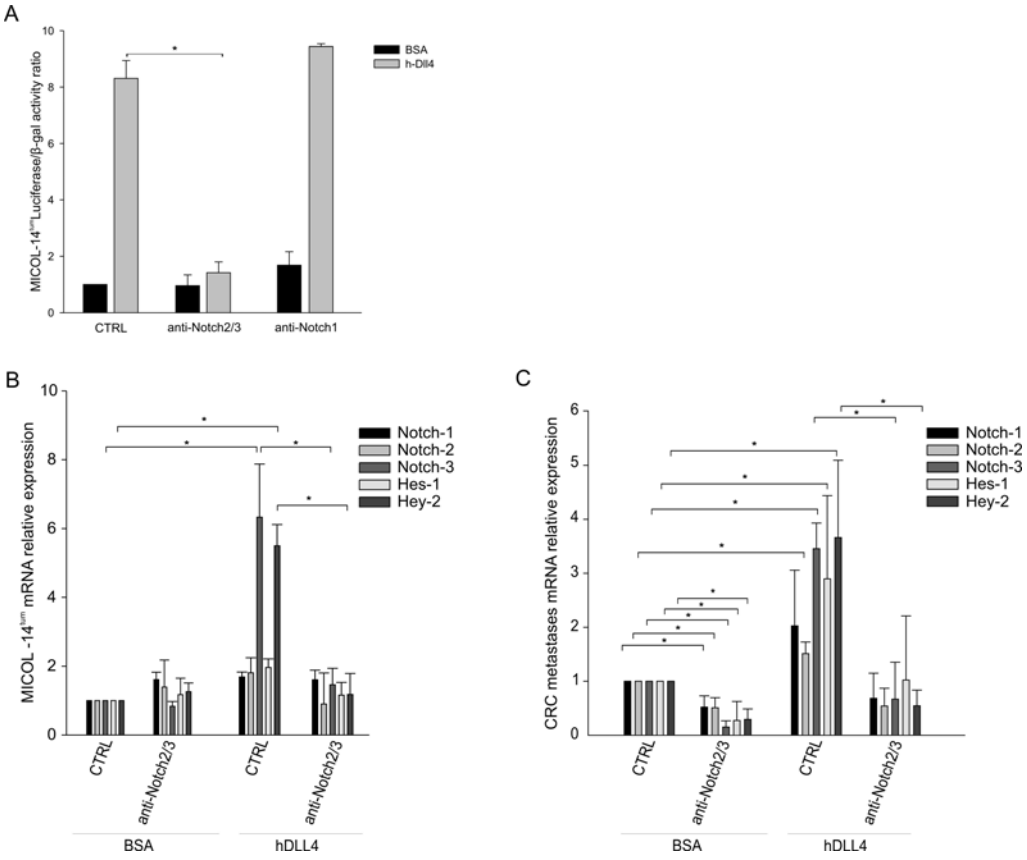

A
MICOL-14^lum Luciferase/β-gal activity ratio
BSA
h-Dll4
CTRL
anti-Notch2/3
anti-Notch1
B
MICOL-14^lum mRNA relative expression
Notch-1
Notch-2
Notch-3
Hes-1
Hey-2
CTRL
anti-Notch2/3
CTRL
anti-Notch2/3
BSA
hDLL4
C
CRC metastases mRNA relative expression
Notch-1
Notch-2
Notch-3
Hes-1
Hey-2
CTRL
anti-Notch2/3
CTRL
anti-Notch2/3
BSA
hDLL4

Figure S2

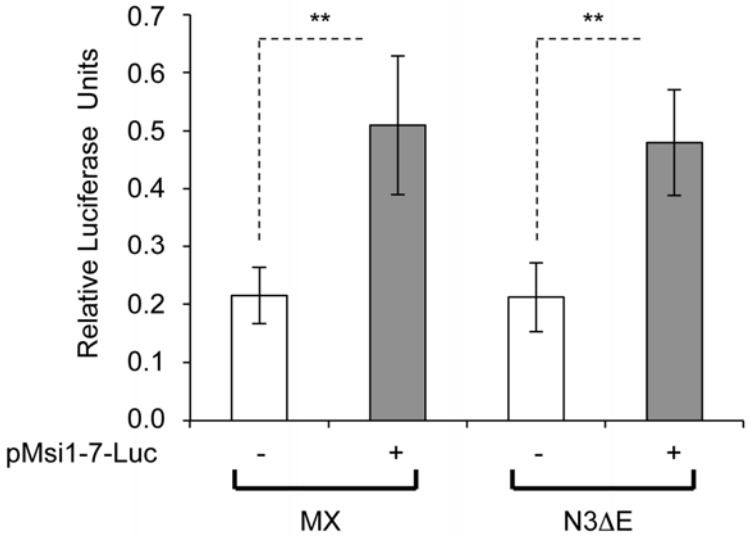

Relative Luciferase Units
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
**
pMsi1-7-Luc
-
+
-
+
MX
N3ΔE

Table S1: primer sequences

Gene name	Forward	Reverse
Notch-1	5'-GTGCCCTCATGGACGACAAC-3'	5'- GCATCCAGGTGCTGCTGAGT-3'
Notch-2	5'-CAGCCTGTATGTGCCCTGTG-3'	5'-GTGCTCCCTTCAAAACCTGCA-3'
Notch-3	5'-CAAGGGTGAGAGCCTGATGG-3'	5'- GAGTCCACTGACGGCAATCC-3'
Hes-1	5'-CAGCGGGCGCAGATGAC-3'	5'-CGTTCATGCACTCGCTGAAG-3'
Hey-2	5'-GGCGTCGGGATCGGATAAAT-3'	5'-GCGTGTGCGTCAAAGTAGCC-3'
Msi-1	5’-GGTTTCCAAGCCACAACCTA-3’	5’-TCGGGGAACTGGTAGGTGTA-3’
Muc-1	5'-AGTTCAGGCCAGGATCTGTG-3'	5'-GAAATGGCACATCACTCACG-3'
Muc-2	5-'ACCCGCACTATGTCACCTTC-3'	5'-GGGATCGCAGTGGTAGTTGT-3'
Numb	5'-AGCCAGCCCATACTGCTCTA-3'	5'-CGGACGCTCTTAGACACCTC-3'
CK20	5’-GACGCCAGAACAACGAATACC-3’	5’-ACGACCTTGCCATCCACTAC-3’
β2micro	5’-TCTCTCTTTCTGGCCTGGAG-3’	5’-TCTCTGCTGGATGACGTGAG-3’
ALDH1A	5’-CCCGTTGGTTATGCTCATTT-3’	5’-TGCTCTGCTGGTTTGACAAC-3’