Silencing of the EPHB3 tumor-suppressor gene in human colorectal cancer through decommissioning of a transcriptional enhancer

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Edited by Leonard I. Zon, Howard Hughes Medical Institute, Boston Children's Hospital and Harvard Medical School, Boston, MA, and accepted by the Editorial Board February 24, 2014 (received for review July 31, 2013)

The protein tyrosine kinase Ephrin type-B receptor 3 (EPHB3) counteracts tumor-cell dissemination by regulating intercellular adhesion and repulsion and acts as tumor/invasion suppressor in colorectal cancer. This protective mechanism frequently collapses at the adenoma–carcinoma transition due to *EPHB3* transcriptional silencing. Here, we identify a transcriptional enhancer at the EPHB3 gene that integrates input from the intestinal stem-cell regulator achaete-scute family basic helix-loop-helix transcription factor 2 (ASCL2), Wnt/β-catenin, MAP kinase, and Notch signaling. *EPHB3* enhancer activity is highly variable in colorectal carcinoma cells and precisely reflects *EPHB3* expression states, suggesting that enhancer dysfunction underlies *EPHB3* silencing. Interestingly, low Notch activity parallels reduced *EPHB3* expression in colorectal carcinoma cell lines and poorly differentiated tumor-tissue specimens. Restoring Notch activity reestablished enhancer function and *EPHB3* expression. Although essential for intestinal stem-cell maintenance and adenoma formation, Notch activity seems dispensable in colorectal carcinomas. Notch activation even promoted growth arrest and apoptosis of colorectal carcinoma cells, attenuated their self-renewal capacity in vitro, and blocked tumor growth in vivo. Higher levels of Notch activity also correlated with longer disease-free survival of colorectal cancer patients. In summary, our results uncover enhancer decommissioning as a mechanism for transcriptional silencing of the *EPHB3* tumor suppressor and argue for an antitumorigenic function of Notch signaling in advanced colorectal cancer.

tumor progression | metastasis | Ephrin signaling | EPHB2

In colorectal tumorigenesis, signaling by the protein tyrosine kinases Ephrin type-B receptor 2 (EPHB2) and 3 (EPHB3) represents a powerful barrier against tumor-cell spreading and the onset of metastasis, the main cause for cancer-related mortality (1, 2). Through repulsive interactions between cells expressing EPHB receptors and cells presenting EphrinB ligands, EPHB/EphrinB signaling compartmentalizes tumors and locally confines their growth (3). In addition, EPHB/EphrinB signaling affects the function of the cell–cell adhesion molecule E-cadherin, thereby adding to the stabilization of a noninvasive epithelial-cell phenotype (3). However, colorectal carcinomas frequently overcome the invasion/tumor suppressor function of the EPHB/EphrinB system by transcriptional down-regulation of receptor expression (1, 2, 4).

Transcriptional silencing of tumor-suppressor genes is often ascribed to inappropriate methylation of promoter DNA and the occurrence of posttranslational modifications of core histones that specify transcriptionally repressed states (5). However, regulation of eukaryotic gene expression is a complex process and involves several other classes of control elements in addition to promoters (6). Principally, malfunction of any of these could lead to faulty gene expression. In this regard, transcriptional enhancers are of particular interest. They vastly outnumber promoter elements and play dominant roles in the coordinated control of gene expression (6, 7). Molecularly, they represent clusters of transcription factor binding sites that allow integrating input from multiple signaling cascades to orchestrate spatiotemporal gene-expression patterns (6). Interestingly, gain-of-function mutations in enhancer DNA have been linked to hyperactivation of the c-MYC proto-oncogene (8), but the involvement of transcriptional enhancers in tumor-suppressor silencing still needs to be explored.

In colorectal tumors, *EPHB3* exhibits biphasic expression profiles that are distinguished by a surge in expression in adenomas and secondary down-regulation in carcinomas (1, 2, 4). *EPHB3* is a direct target of Wnt/β-catenin signaling (9), which may explain *EPHB3* up-regulation in early tumorigenesis. *EPHB3* secondary down-regulation correlates with a loss of histone marks characterizing active gene loci (4), but the molecular basis for this is unknown. We therefore set out to identify regulatory elements and control mechanisms involved in *EPHB3* expression that might become incapacitated in the course of colorectal carcinogenesis. Here, we report the identification and in-depth characterization of a bona fide transcriptional enhancer at the *EPHB3* locus and implicate enhancer dysfunction in *EPHB3* down-regulation. Further analyses showed that Wnt/β-catenin,

Significance

Metastasis—the main cause of cancer-related death—is initiated when cells detach from the primary tumor and invade the surrounding tissue. In colorectal cancer, tissue invasion is facilitated by down-regulation of protein tyrosine kinases from the Ephrin type-B receptor family (EPHB), which function as tumor suppressors by controlling cellular positioning and restricting tumor-cell motility. This study shows that inactivation of the EPHB3 gene results from the incapacitation of a transcriptional enhancer element. *EPHB3* enhancer dysfunction is a consequence of defective Notch signaling, which undergoes a switch in functional importance and becomes dispensable and even tumor suppressive in the course of tumorigenesis. These findings have implications for therapeutic strategies that aim at targeting Notch signaling in colorectal cancer.

Author contributions: S.J., H.A., M.J., T.M., R.Z., S.L., and A.H. designed research; S.J., K.R., S.T., H.A., M.B., M.J., A.P., M.S., A.Y., T.M., and S.L. performed research; S.J., K.R., S.T., H.A., M.B., M.J., A.P., M.S., A.Y., T.M., R.Z., M.W., S.L., and A.H. analyzed data; S.J., K.R., S.T., T.M., R.Z., M.W., S.L., and A.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. L.I.Z. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1314523111/-/DCSupplemental.

Notch, and mitogen-activated protein kinase (MAPK) signaling and the stem cell transcription factor ASCL2 converge on the *EPHB3* enhancer and that a defect in Notch signaling is involved in *EPHB3* silencing. Our results identify decommissioning of a transcriptional enhancer as a mechanism for silencing of a tumor-suppressor gene and reveal a change in the functional importance of Notch signaling, which becomes dispensable and even growth inhibitory in the course of colorectal cancer (CRC) progression.

Results

Identification of a Cell Type-Specific Transcriptional Enhancer in the 5′-Flanking Region of the Human EPHB3 Gene. To identify *cis*-regulatory elements involved in *EPHB3* regulation, we performed in silico analyses exploiting the fact that evolutionary conserved regions (ECRs) often represent DNA elements with regulatory potential (10). Sequence comparison between mouse and human genomes revealed several ECRs within the *EPHB3* upstream region (Fig. 1*A*). Consistent with regulation of *EPHB3* by Wnt/β-catenin signaling, two of the *EPHB3* ECRs harbor known or predicted T-cell factor/lymphoid enhancer factor (TCF/LEF) binding elements (TBEs) (11). The regulatory potential of the ECRs was analyzed by luciferase-reporter assays in a panel of CRC cell lines that harbor characteristic alterations in several tumor suppressors and oncogenes (Fig. S1*A*) and that faithfully reflect the variable levels of *EPHB3* expression seen in human CRC tissue specimens (Fig. 1*B*) (4). In these experiments, the presence of the ECR centered around −2.3 kb strongly increased reporter-gene activity (Fig. 1*C* and Fig. S1*B*). Moreover, the −2.3-kb ECR stimulated the heterologous SV40 promoter in a distance- and orientation-independent manner (Fig. 1*D* and Fig. S1*C*). Importantly, the stimulatory capacity of the −2.3-kb ECR differed widely among the CRC cell lines and was most pronounced in LS174T cells with highest levels of endogenous *EPHB3*. Thus, the *EPHB3* −2.3-kb ECR functions as a cell type-specific transcriptional enhancer. Consistent with its evolutionary conservation, also the corresponding region from the mouse EphB3 gene has enhancer properties and exhibits a remarkably similar cell-type specificity in CRC cell lines (Fig. S1*E*).

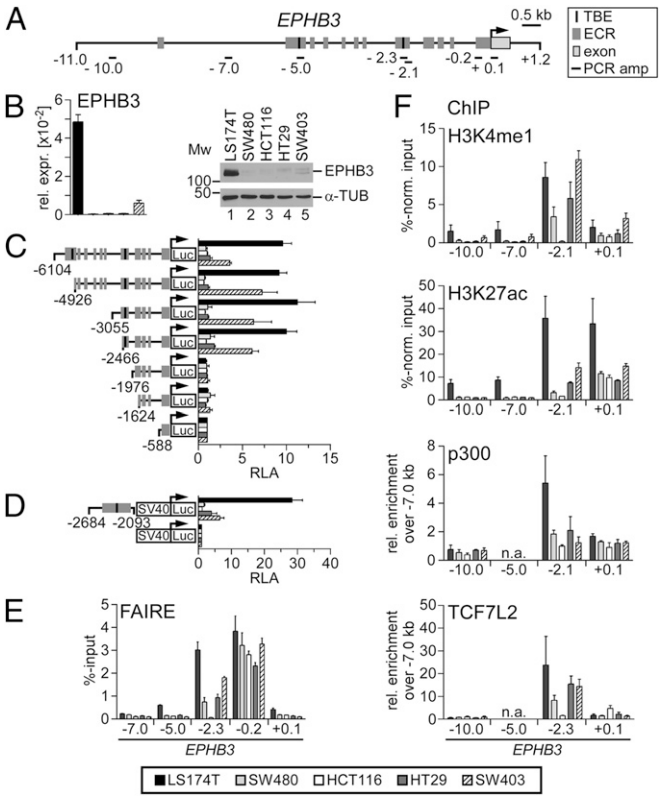

Fig. 1. A cell type-specific transcriptional enhancer at the EPHB3 gene. (*A*) Scheme of the *EPHB3* 5′-flanking region. TBE, TCF/LEF binding element; ECR, evolutionary conserved region. PCR amp, PCR amplicon. (*B*) Quantitative reverse-transcription PCR (qRT-PCR) and Western blot analyses of *EPHB3* expression in CRC cell lines. rel. expr., relative expression; α-TUB, α-TUBULIN immunodetection to monitor equal loading. M_W, molecular mass in kDa. (*C*) Reporter-gene assay with constructs driven by *EPHB3* genomic sequences ($n \geq 3$). RLA, relative luciferase activity. (*D*) Cell type-specific enhancer activity of the *EPHB3* −2.3-kb ECR assayed with the SV40 promoter ($n \geq 3$). (*E*) FAIRE analysis of the *EPHB3* locus ($n = 3$). (*F*) ChIP analyses of H3K4me1, H3K27ac, p300, and TCF7L2 occupancy at the *EPHB3* locus ($n = 3$). rel. enrichment, relative enrichment; %-norm. input, %-input normalized to H3.

Transcriptional enhancers can be inactive, poised, or active. These functional states can be distinguished according to the chromosomal accessibility of enhancer DNA, the binding of transcription factors, and the occurrence of characteristic patterns of histone modifications (6, 7). We probed these features by chromatin immunoprecipitation (ChIP) and formaldehyde-assisted isolation of regulatory elements (FAIRE) (12). By FAIRE, the *EPHB3* −2.3-kb enhancer region exhibited differential accessibility, with partially or completely closed chromatin in CRC cells with low *EPHB3* expression levels (Fig. 1*E*). Similarly, the mouse *EphB3* enhancer region displayed a more open chromatin structure in EphB3-positive intestinal crypts compared with EphB3-negative villi (Fig. S1 *F* and *G*). In contrast, human and mouse *EPHB3* promoter sequences (probe location −0.2 kb) were strongly enriched from all cell lines and both crypt and villus fractions, respectively, arguing for an open chromatin conformation irrespective of *EPHB3* expression levels (Fig. 1*E* and Fig. S1*G*). Thus, differences in chromatin structure at the *EPHB3* −2.3-kb enhancers more closely reflect differences in *EPHB3* transcriptional activity.

Next, we performed ChIP experiments to investigate the distribution of the histone modifications H3K4me1 and H3K27ac, the acetyltransferase p300, and the Wnt pathway effector TCF7L2 at the *EPHB3* locus. H3K4me1 is a characteristic of poised enhancers whereas H3K27ac and p300 are features of active enhancers (7). At *EPHB3*, especially, indicators of active enhancers (H3K27ac, p300) and occupancy by TCF7L2 showed striking differences among the CRC cell lines and were again most pronounced in LS174T cells (Fig. 1*F*). Differential binding of TCF7L2 to the *EPHB3* locus is not due to pathway defects in some cell lines because TCF7L2 could be detected at other Wnt/β-catenin target genes (Fig. S1*D*). In summary, the *EPHB3* −2.3-kb ECR has functional properties and structural hallmarks of a bona fide transcriptional enhancer. Significantly, its cell type-specific activity matches the expression of the endogenous EPHB3 gene in CRC cell lines, suggesting that differences in enhancer function underlie differential *EPHB3* expression in CRC.

Combinatorial Control of EPHB3 Expression and Enhancer Activity by Wnt/β-Catenin, Notch, and MAPK Signaling and the Stem-Cell Factor ASCL2. *EPHB3* expression is restricted to intestinal stem cells (ISCs), transit amplifying cells, and Paneth cells located in the lower part of intestinal crypts (9, 13). The maintenance and generation of these cell populations requires the coordinated activity of the Wnt/β-catenin, Notch, and MAPK signal-transduction cascades (14). Previously, separate reports showed that *EPHB3* can be regulated by Wnt/β-catenin and Notch signaling and that TCF7L2 and the Notch intracellular domain (NICD) (an indicator of Notch receptor activation) bind to the *EPHB3* enhancer region (11, 15). Furthermore, the RBPJ site and the TBE are flanked by an E-box motif that is occupied by the ISC transcription factor ASCL2 (Fig. 2*A* and Fig. S2) (16). However, the close proximity of these binding sites and the functional importance of the *EPHB3* −2.3-kb region were not realized before. Further sequence analyses of the *EPHB3* enhancer region showed the presence of potential recognition motifs for members of the ETS family of transcription factors (Fig. 2*A* and Fig. S2), which play important roles in the development and differentiation of

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the intestinal epithelium and colorectal tumorigenesis and can be downstream of MAPK signaling (17–19). These observations suggest that the major crypt base signaling cascades and transcription factors converge on the *EPHB3* enhancer to control *EPHB3* expression. To investigate this, we used shRNA-mediated knock-down of β-catenin and small-molecule inhibitors of MEK (U0126) and γ-secretase (DAPT) to individually inhibit Wnt/β-catenin, MAPK, and Notch signaling, respectively. Alternatively, Notch signaling was inhibited by doxycycline (Dox)-induced expression of dominant-negative mastermind-like 1 (dnMAML1). All treatments resulted in reduced expression of *EPHB3* and control genes (Fig. S3 *A–D*), confirming the individual roles of Wnt/β-catenin, MAPK, and Notch signaling in the regulation of *EPHB3*. Next, we combined Dox-inducible TCF7L2 knockdown with U0126 and/or DAPT treatment (Fig. 2*B* and Fig. S3*E*). Blocking Wnt/β-catenin, Notch, and MAPK signaling in a pairwise fashion additively repressed *EPHB3* expression. The greatest reduction in *EPHB3* expression was obtained when all three pathways were inhibited simultaneously. Thus, high-level *EPHB3* expression entails the cooperation of Wnt/β-catenin, Notch, and MAPK signaling.

Next, we generated luciferase-reporter constructs with point mutations in the TBE and the binding sites for RBPJ and ETS factors (Fig. 2*A*) to examine their importance for *EPHB3* enhancer function. Constructs with a mutation in the E-box were included to address the role of ASCL2. Furthermore, we split the enhancer into subfragments that separated the ETS and RBPJ binding motifs, the TBE, and the E-box from each other (Fig. S4*A*) and analyzed the transcriptional activity of the subfragments and their combinations, as well as their response to inhibition of Wnt/β-catenin, Notch, and MAPK signaling (Fig. S4 *B* and *C*). Overall, we found that full enhancer activity requires at least the RBPJ site, the proximal ETS binding motif, the TBE, and the E-box containing subfragment (Fig. 2*A* and Fig. S4*C*). As expected, the TBE and RBPJ binding motifs seem to be critical in mediating Wnt and Notch responses whereas several enhancer subfragments integrate the input from MAPK signaling.

ASCL2 binds to the E-box at the *EPHB3* enhancer in vitro (Fig. S4*D*) and in vivo (16), yet disrupting the E-box seemingly did not impair the function of the isolated *EPHB3* enhancer.

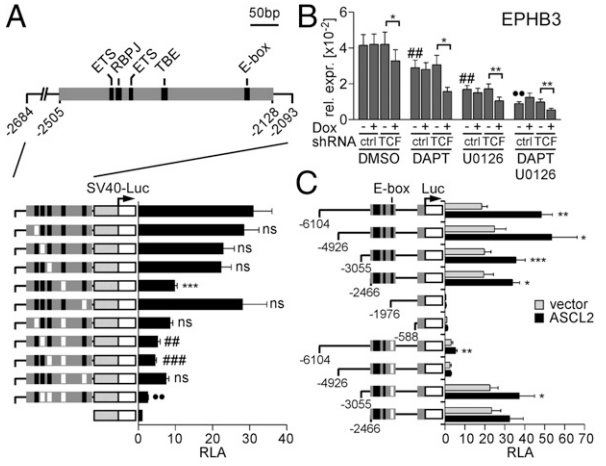

Fig. 2. The *EPHB3* enhancer receives input from major crypt base signaling pathways. (*A*) Predicted transcription factor binding sites at the *EPHB3* enhancer and reporter-gene assay to test their functional importance ($n \geq 4$). *, compared with wild type; #, compared with TBE mutant; •, compared with TBE/RBPJ and TBE/ETS II mutants; ns, not significant. (*B*) qRT-PCR to analyze relative expression (rel. expr.) of *EPHB3* in LS174T CRC cells upon inhibition of Notch (DAPT), MAPK (U0126), and Wnt/β-catenin signaling (shTCF7L2) ($n = 6$). *, compared with Dox; #, compared with DMSO; •, compared with DAPT and U0126 (paired, two-tailed Student *t* test). (*C*) Luciferase-reporter assay to test the roles of ASCL2 and the *EPHB3* enhancer E-box ($n = 6$) (paired, two-tailed Student *t* test). RLA, relative luciferase activity.

Nonetheless, overexpression of ASCL2 stimulated *EPHB3* luciferase-reporter activity (Fig. 2*C*), and mutation of the E-box diminished activity of reporter constructs with extended *EPHB3* sequences. Thus, binding of ASCL2 seems to play a role in the communication between the enhancer and more distal control elements. In summary, the results of these studies together with the inhibitor experiments and *EPHB3* expression analyses, demonstrate that major crypt base signaling cascades and transcription factors converge on the *EPHB3* enhancer and cooperate to control *EPHB3* expression.

EPHB2 is a paralogue of *EPHB3* that also undergoes secondary silencing in CRC (1, 4). When we applied the experimental strategy used for the identification and characterization of the *EPHB3* −2.3-kb enhancer, we found that differential expression of *EPHB2* in CRC cells also seems to be determined by a cell type-specific enhancer element that is located around −8.4 kb upstream of the *EPHB2* promoter (Fig. S5). Despite the involvement of transcriptional enhancers in the (de-)regulation of both genes, *EPHB2* and *EPHB3* regulation differs in mechanistic details. For example, neither knock-down of TCF7L2 nor inhibition of Notch and MAPK signaling affected *EPHB2* expression (Fig. S3*F*), and the *EPHB2* and *EPHB3* enhancers showed diverging activities in SW403 cells (compare Fig. 1*D* and Fig. S5*D*). Nonetheless, the EPHB2 gene provides an additional example for tumor-suppressor silencing by enhancer decommissioning.

Notch Deficiency Impairs EPHB3 Enhancer Function. Characterization of *EPHB3* enhancer mutants revealed that mutating the RBPJ binding motif had opposite effects on *EPHB3* enhancer activity in LS174T and HT29 cells (Fig. S4*E*). This behavior is in agreement with the dual function of RBPJ as transcriptional repressor and activator depending on the functional state of Notch signaling and suggested that Notch signaling is inactive in some CRC cells. Indeed, despite comparable receptor expression, levels of NICD differed among the CRC cells under investigation (Fig. 3*A*). Significantly, HT29 cells with the lowest amounts of NICD had the lowest expression levels of Notch ligands. Furthermore, expression of Notch target genes varied considerably in our panel of CRC cells (Fig. 3*B*). Overall, the expression profile of Notch target genes suggests that the Notch pathway is active in LS174T cells but has little or no activity in the other cell lines.

To test whether restoring Notch activity would increase *EPHB3* expression, we activated the Notch pathway in HT29 cells by stimulation with recombinant Dll1 or by Dox-induced expression of HA-tagged NICD. Dll1 treatment increased NICD levels and HES1 expression (Fig. 3*C*). Dox induction of NICD also stimulated HES1 expression (Fig. 3*D*). Moreover, Dll1 treatment and expression of NICD strongly increased *EPHB3* expression (Fig. 3 *C* and *D*), albeit without affecting *EPHB2*, confirming that the two genes are regulated by distinct mechanisms. A stimulatory effect on *EPHB3* was also obtained with SW403 CRC cells that resemble HT29 in terms of low *EPHB3* expression and Notch activity (Fig. S6). ChIP experiments confirmed that reconstituted Notch signaling directly targets the *EPHB3* enhancer (Fig. 3*E*) although its overall structure did not change (Fig. S7*A*). Furthermore, in reporter-gene assays, expression of NICD stimulated *EPHB3* enhancer activity depending upon an intact RBPJ binding site (Fig. S7*B*). Taken together, these results establish Notch-pathway deficiency as a cause for impaired *EPHB3* enhancer function and reduced *EPHB3* expression in CRC cell lines.

EPHB3 Expression and Notch Activity Are Positively Correlated in Human-Tissue Specimens. To substantiate the pathophysiological relevance of the regulatory relationship between Notch signaling and *EPHB3*, we analyzed publicly available gene expression data from The Cancer Genome Atlas (TCGA) and performed pairwise correlation analyses. Consistent with the results of our expression screening in CRC cell lines, this analysis showed that the expression of *EPHB3* is positively correlated with several Notch ligands, receptors, and target genes: e.g., *DLL1*, *DLL4*, *NOTCH1, HES1*, *HES5*, and *HES6* (Fig. 4*A*). We also conducted

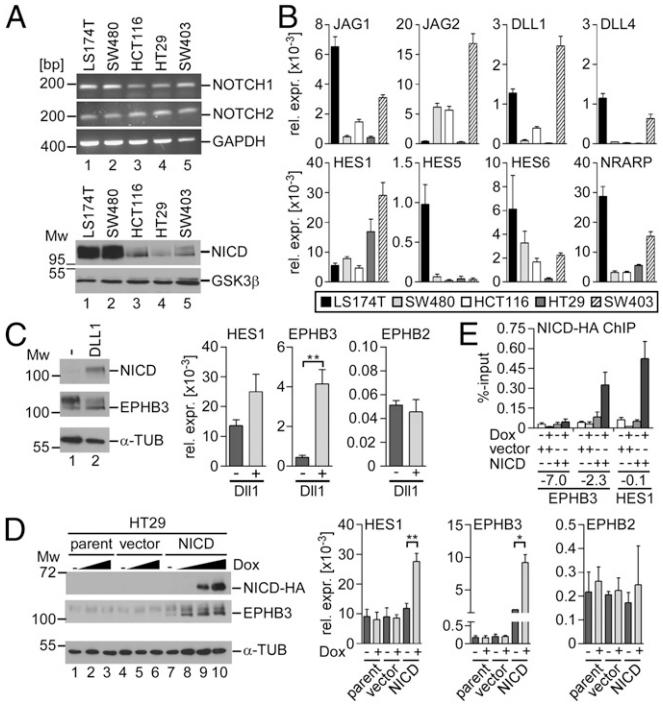

Fig. 3. Defective Notch signaling contributes to inactivation of the *EPHB3* enhancer. (*A*) RT-PCR analyses of *NOTCH* expression and Western blot analyses of NICD abundance in CRC cell lines. (*B*) qRT-PCR to analyze relative expression (rel. expr.) of Notch ligands and target genes ($n = 3$). (*C* and *D*) Western blot analyses of NICD and EPHB3 and qRT-PCR analyses of *HES1*, *EPHB2*, and *EPHB3* in (*C*) HT29 CRC cells treated with Dll1 ($n = 4$) and (*D*) in parental (parent) cells and derivatives stably transduced with Dox-inducible retroviral control or NICD-HA expression vectors ($n = 3$). (*E*) ChIP analyses of NICD-HA occupancy at *EPHB3* and *HES1* in HT29 CRC cells and derivatives ($n = 3$). (*A*, *C*, and *D*) GSK3β or α-TUBULIN (α-TUB) immunodetection to monitor equal loading. M_W = molecular mass in kDa.

immunohistochemical analyses of EPHB3 and NICD in human CRC tissue specimens. When stratified according to histological grading, we found that the vast majority of moderately differentiated grade 2 tissue specimens stained positive for both EPHB3 and NICD (Fig. 4 *B–D* and Table S1) whereas poorly differentiated grade 3 tumor specimens were mostly negative for both proteins. The difference in expression between grades 2 and 3 was highly significant for EPHB3 ($P = 0.001$) and showed a near significant trend for NICD ($P = 0.07$). The expression of EPHB3 and NICD was clearly correlated among the samples ($P = 0.005$). This strikingly parallel behavior of EPHB3 and NICD expression further supports a regulatory relationship between Notch activity and *EPHB3* expression in human tumors.

Furthermore, we defined a gene set consisting of *EPHB3* and other well-characterized Notch or combined Notch/Wnt target genes that strongly responded to Notch activation in our CRC cell line model (15, 20, 21) (Fig. 5*D*) and examined disease-free survival in relation to expression levels of this gene set based on transcriptome data of 197 CRC patients (22). This analysis revealed that higher expression levels of *EPHB3* and other genes indicative of an active Notch pathway have a significantly better prognosis (Fig. 4*E*).

Notch Activation Causes Cell-Cycle Arrest and Induces Apoptosis. Notch signaling is thought to be oncogenic in CRC (23, 24). However, there is evidence that Notch signaling impairs CRC progression (20), and absence of Notch activity has been observed in human carcinomas (ref. 23 and Fig. 4). Therefore, we wondered about the significance of Notch signaling in CRC cells and inhibited the pathway in LS174T cells by DAPT or expression of dnMAML1. However, this had no adverse effects on LS174T population dynamics (Fig. S8 *A* and *B*) even though Notch is active in these cells. More strikingly, growth of four CRC cell lines with low Notch activity (Fig. 3 and Fig. S8 *G* and *H*) was reduced upon NICD expression (Fig. 5*A* and Fig. S8 *C–E*). This was accompanied by up-regulation of the CDK inhibitor $CDKN1A/p21^{CIP1}$ (Fig. 5*D* and Fig. S8*F*) and G1/S cell-cycle arrest (Fig. 5*B*). Furthermore, NICD expression induced apoptosis in HT29 cells as detected by increased levels of cleaved CASPASE3 and its substrate PARP1 (Fig. 5*C*). The growth inhibitory effect of Notch activation was also observed in vivo upon injection of HT29 control and NICD-expressing cells into immunodeficient mice. Following Dox application, development of tumors from NICD-expressing cells was strongly retarded (Fig. 6). Together, these experiments demonstrate that Notch activity can be dispensable for CRC cells and that forced activation of the Notch pathway can even halt CRC cell proliferation in vitro and tumor growth in vivo.

Notch Activation Impairs Stemness of CRC Cells. Intestinal stem or stem-like cells are thought to be the cells of origin in CRC (25), and Notch activity is essential for ISC maintenance and adenoma formation (21, 24, 26, 27), which contrasts with the observed heterogeneity and absence of Notch activity in colorectal carcinomas. Therefore, we performed additional gene-expression analyses to further characterize phenotypic consequences of Notch activation. Marker genes for tumor-initiating cells were largely unaffected by NICD expression (Fig. S9*A*), but *OLFM4*, a known Notch target (21), and the differentiation markers *CDX1*, *CDX2* and *KRT20* (28–30) were up-regulated (Fig. 5*D*). The latter suggests that NICD expression initiates lineage commitment and enterocytic differentiation. Consistent with this, we observed that NICD expression led to a marked down-regulation of the ISC markers *LGR5* and *ASCL2* (Fig. 5*D*) and strongly impaired the self-renewal capacity of HT29 cells in a sphere formation/serial limiting dilution assay, providing functional evidence for a loss of stem-cell properties (Fig. 5*D* and Fig. S9*B*). We also interrogated TCGA gene-expression data for correlations between

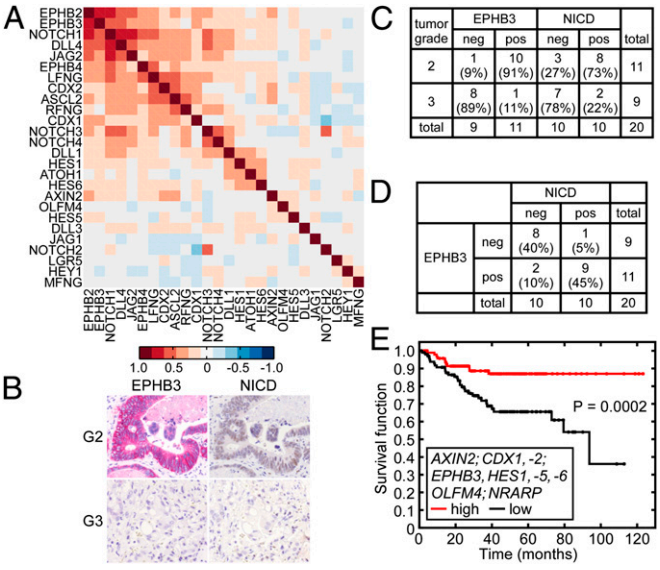

Fig. 4. Highly correlated expression of *EPHB3* and Notch activity in human CRC tissue specimens. (*A*) Pairwise correlation analyses of gene expression based on TCGA data. Color scale reflects the correlation coefficient as indicated by the color bar. (*B*) Immunohistochemical analyses of EPHB3 and NICD expression in sections of human CRC tissue specimens representing grade 2 (G2) and 3 (G3) tumors. (*C*) Frequency of EPHB3 and NICD expression in human CRC tissue specimens. (*D*) Significantly correlated ($P = 0.005$; Fisher's exact test) coexpression of EPHB3 and NICD in human CRC tissue specimens. (*E*) Disease-free survival of CRC patients with high or low expression of the indicated gene set ($P = 0.0002$; logrank test).

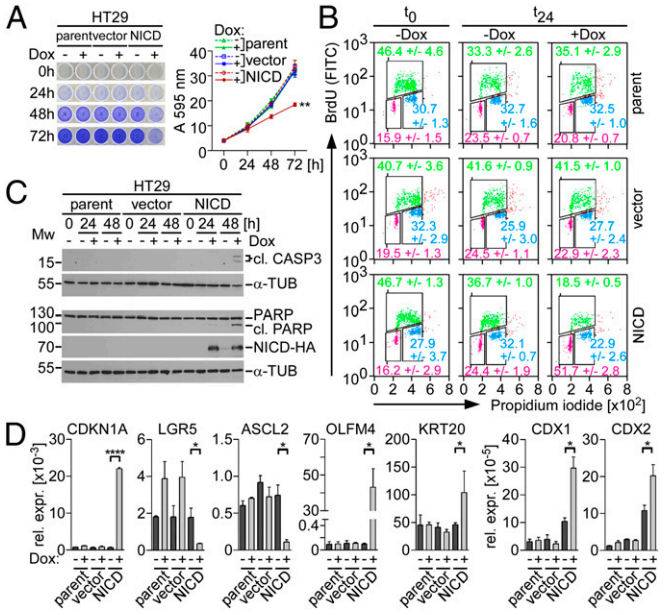

Fig. 5. Notch activation causes cell-cycle arrest, differentiation, and apoptosis of CRC cells. (*A*) Crystal-violet stainings and corresponding quantifications to assess population dynamics of parental (parent) HT29 CRC cells and their derivatives ($n = 3$) (two-way ANOVA). (*B*) Cell-cycle analysis using flow cytometry after BrdU/propidium iodide staining. Cell populations in G1, S, and G2/M cell-cycle phases are colored pink, green, and blue, respectively ($n = 3$). (*C*) Western blot analyses of cleaved CASPASE3 and PARP1 at different time points after Dox treatment. α-TUB, α-TUBULIN immunodetection to monitor equal loading; M_W, molecular mass in kDa. (*D*) qRT-PCR analyses to assess relative expression (rel. expr.) of the genes indicated ($n = 3$).

the expression of Notch-pathway components *ASLC2*, *LGR5*, *OLFM4*, *CDX1*, and *CDX2* (Fig. 4). Indeed, expression of *CDX1* and *CDX2* was positively correlated with Notch-pathway components and Notch target genes also in human tumors (Fig. 4). Together with the results obtained with CRC cells and from the immunohistochemical analyses of tissue specimens, these findings further support the idea that, in colorectal carcinomas, Notch activity impairs stemness features and is linked to a higher degree of tumor-cell differentiation.

Discussion

In CRC, *EPHB2* and *EPHB3* are important tumor suppressors whose activity effectively restricts tumor-cell spreading (1, 2). However, the transition from noninvasive adenoma to invasive carcinoma states is frequently paralleled by their transcriptional down-regulation (1, 2, 4). The heterogeneity in *EPHB2*/*EPHB3* expression observed in primary cancer tissues is accurately reflected in our CRC cell line model and provided a basis for determining molecular mechanisms that govern *EPHB2*/*EPHB3* expression. For both genes we identified transcriptional enhancers that show marked differences in transcription-factor binding, chromatin structural hallmarks, and functionality within our CRC cell line model. Importantly, different functional states of the *EPHB2*/*EPHB3* enhancer elements are a precise projection of *EPHB2*/*EPHB3* transcriptional activity in CRC cell lines. Similarly, we implicate the murine element in the physiological regulation of *EphB3* in the intestine. We therefore propose that the *EPHB2*/*EPHB3* enhancer elements play key roles in the regulation of *EPHB2*/*EPHB3* expression and that enhancer dysfunction accounts for *EPHB2*/*EPHB3* secondary silencing during tumor progression.

EPHB2 and *EPHB3* show similar expression changes during colorectal-cancer progression (1, 4), and secondary down-regulation of both genes seemingly involves the inactivation of promoter-distal enhancers. Nonetheless, this study and previous work showed that molecular details of *EPHB2*/*EPHB3* inactivation differ (4). Wnt/β-catenin activity is one of the drivers of *EPHB2*/*EPHB3* expression and likely accounts for their up-regulation in colorectal adenomas (31). Secondary silencing of *EPHB2* and *EPHB3*, however, seems to result from disturbances in regulatory mechanisms that cooperate with Wnt/β-catenin signaling to control *EPHB2*/*EPHB3* expression but differ between the two genes. We speculate that the diverging behavior reflects differences in the signaling pathways that direct expression of *EPHB2* and *EPHB3* within intestinal crypts where *EPHB2* and *EPHB3* are expressed in overlapping but distinct cell compartments (9).

Intestinal-tissue homeostasis requires a complex interplay of Wnt/β-catenin, Notch, and MAPK signaling, which are the predominant pathways that regulate proliferation, lineage decisions, and differentiation in the intestinal epithelium (14). Molecular analyses of its architecture revealed that the *EPHB3* enhancer receives and computes input from all these pathways and the ISC factor ASCL2 and, thus, is ideally designed to function as a signal integrator element with the capacity to drive *EPHB3* expression in diverse cell types that populate intestinal crypts (9, 13). In this regard, the additive rather than synergistic cooperation of transcriptional activators acting upon the enhancer is noteworthy. The consequences of this are incremental changes in enhancer activity and thus *EPHB3* expression even if the activity of one of the activators changes. This type of enhancer design likely represents an adaptation to the need of maintaining adequate levels of *EPHB3* expression in multiple cellular backgrounds exposed to different intensities and combinations of signaling activity.

Molecular dissection of the *EPHB3* enhancer revealed that a deficiency in Notch activity contributes to impaired enhancer function. Despite its transcriptional effect, however, restoring Notch activity did not alter chromatin structure at the *EPHB3* enhancer in HT29 cells. It is possible that chromatin opening requires the activity of pioneer factors and master regulators of lineage commitment, which seem to direct effectors of developmental signaling cascades to their genomic target sites (6, 32). Further examination of regulatory sequences should identify candidate transcription factors that govern chromatin dynamics at the *EPHB3* enhancer.

Notch activity is essential for adenoma formation in mouse tumor models which led to the suggestion that targeting Notch signaling could be a viable antitumor strategy (23, 24). However, there is also evidence that Notch signaling impairs tumor progression and is not strictly required for tumorigenesis in mice (20, 33). Similarly, reduced expression of Notch target genes has been observed in human carcinomas (20, 23). Our results confirm and extend these findings. We show that Notch activity seems to be dispensable in CRC cells and high-grade tumors, that higher expression levels of Notch target genes are associated with longer disease-free survival in CRC, and that Notch activation can even be tumor-suppressive. Antitumorigenic Notch activity could be due to up-regulation of the cell-cycle inhibitor *CDKN1A* and intestinal differentiation markers, and the impaired self-renewal capacities of CRC cells upon Notch activation. Under physiological conditions, the Notch pathway is integrated into a carefully balanced signaling circuitry that optimally sustains the dynamics of cellular proliferation and multilineage differentiation (14, 34). The observed change in the functional impact of

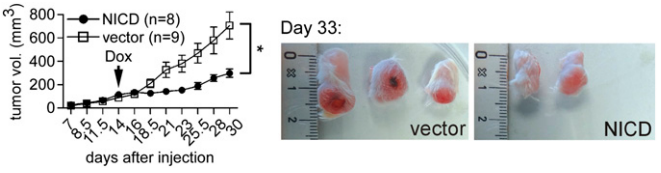

Fig. 6. Notch activation decelerates tumor growth in vivo. Rag2$^{-/-}\gamma c^{-/-}$ mice were injected s.c. with derivatives of HT29 CRC cells ($P < 0.05$; two-way ANOVA). Pictures show representative tumors isolated on day 33.

Notch signaling in carcinomas, compared with adenomas and the healthy intestine, indicates that these signaling networks undergo extensive rewiring in tumorigenesis. Acquired Notch independence or inactivation could be advantageous for tumor cells by alleviating the differentiation-inducing capacity of Notch signaling and by abrogating known antagonistic effects of Notch signaling on Wnt/β-catenin activity (20, 24, 26, 35). In any case, therapies aiming at inhibition of the Notch pathway may prove ineffective or even counterproductive without careful evaluation of the status of the Notch pathway in a particular tumor.

In summary, our findings provide detailed mechanistic insights into the process of tumor-suppressor silencing and raise the possibility that transcriptional enhancers, rather than promoter regions, are the primary targets for molecular mechanisms that deregulate gene expression in tumorigenesis. Such a role agrees very well with the general importance of transcriptional enhancers in gene regulation (6, 7). Moreover, enhancer decommissioning has already been recognized as a regulatory principle during differentiation processes under physiological conditions (36). The ability to precisely delineate defects in regulatory networks that lead to enhancer dysfunction, as demonstrated for the *EPHB3* enhancer, will offer new opportunities to devise therapeutic strategies that aim at rectifying gene-expression patterns and cellular behavior in tumorigenesis.

Materials and Methods

Detailed information about procedures and a list with primers used for qRT-PCR, ChIP and FAIRE (Table S2) can be found in *SI Materials and Methods*.

Manipulation of Notch and MAPK Signaling. Notch signaling was activated with recombinant mouse Dll1-human Fc for 24 h. Notch signaling was blocked by application of 10 μM *N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester (DAPT) for 48 h (single treatment) or 3 h (combinatorial treatment). MAPK signaling was blocked by adding 10 μM U0126 for 3 h before harvest.

Tumorigenesis. $Rag2^{-/-}\gamma c^{-/-}$ mice (37) were injected s.c. with 1×10^6 CRC cells. Tumor size (millimeters) was retraced by measuring tumor diameter with a micrometer. The animal protocol (G08-8) was approved by the Regierungspräsidium Freiburg (local animal committee Freiburg).

Tissue Specimens and Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue specimens of 22 patients with sporadic colorectal carcinomas were analyzed after consent from the local ethics committee (Ethik Kommission, University of Freiburg, approval 324). Serial sections (3 μm) were stained for EPHB3 and NICD following established procedures. Evaluation of EPHB3 and NICD protein abundance was as follows: score 0, negative expression/weakly positive expression in ≤10% of tumor cells; score 1, positive expression in >10% of tumor cells.

Pairwise Correlation Analyses of Microarray Gene-Expression Data. The pairwise Pearson correlation coefficient between 26 genes of interest was calculated from normalized RNA-Seq gene-expression data in 270 colon/rectal cancer samples obtained from The Cancer Genome Atlas. Data tables (release date February 2, 2012) were downloaded from https://tcga-data.nci.nih.gov/docs/publications/coadread_2012/. Genes were clustered in the pairwise correlation matrix using single linkage with Euclidean distance.

Statistical Analysis. For all measurements the mean ± SEM is given. To determine statistical significance an unpaired, two-tailed Student *t* test was used unless otherwise indicated. Numbers of asterisks or corresponding symbols represent statistically significant changes with the following *P* values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

ACKNOWLEDGMENTS. We thank T. Brabletz, T. Borggrefe, and C. Dive for the gift of plasmids and cell lines, and K. Geiger and N. Bittermann for excellent technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft [DFG CRC-850 subprojects B5 (to A.H.), C5 and Z1 (to S.L. and M.W.), and Z2 (to R.Z.)]. K.R. was a fellow of the Studienstiftung des deutschen Volkes. S.T. and H.A. held DFG CRC-850 GEROK positions.

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