Crosstalk between the Notch and wingless-type MMTV integration site (WNT) signaling pathways has been investigated for many developmental processes. However, this negative correlation between Notch and WNT/β-catenin signaling activity has been studied primarily in normal developmental and physiological processes in which negative feedback loops for both signaling pathways are intact. We found that Notch1 signaling retained the capability of suppressing the expression of WNT target genes in colorectal cancers even when β-catenin destruction by the adenomatous polyposis coli (APC) complex was disabled. Activation of Notch1 converted high-grade adenoma into low-grade adenoma in an Apc\textsuperscript{min} mouse colon cancer model and suppressed the expression of WNT target genes in human colorectal cancer cells through epigenetic modification recruiting histone methyltransferase SET domain bifurcated 1 (SETDB1). Extensive microarray analysis of human colorectal cancers also showed a negative correlation between the Notch1 target gene, Notch-regulated ankyrin repeat protein 1 (NRARP), and WNT target genes. Notch is known to be a strong promoter of tumor initiation, but here we uncovered an unexpected suppressive role of Notch1 on WNT/β-catenin target genes involved in colorectal cancer.
Notch1 counteracts WNT/β-catenin signaling through chromatin modification in colorectal cancer

Hyun-A Kim,1 Bon-Kyoung Koo,1 Ji-Hoon Cho,2 Yoon-Young Kim,1,3 Jinwoo Seong,1 Hee Jin Chang,4 Young Min Oh,3 Daniel E. Stange,5 Jae-Gahb Park,4 Dahee Hwang,2 and Young-Yun Kong1

1Department of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul, Republic of Korea. 2School of Interdisciplinary Bioscience and Bioengineering, and 3Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang, Republic of Korea. 4Research Institute and Hospital, National Cancer Center, Goyang, Gyeonggi, Republic of Korea. 5Hubrecht Institute-KNAW (Royal Netherlands Academy of Arts and Sciences) and University Medical Center Utrecht, Utrecht, The Netherlands.

Crosstalk between the Notch and wingless-type MMTV integration site (WNT) signaling pathways has been investigated for many developmental processes. However, this negative correlation between Notch and WNT/β-catenin signaling activity has been studied primarily in normal developmental and physiological processes in which negative feedback loops for both signaling pathways are intact. We found that Notch1 signaling retained the capability of suppressing the expression of WNT target genes in colorectal cancers even when β-catenin destruction by the adenomatous polyposis coli (APC) complex was intact. Activation of Notch1 converted high-grade adenoma into low-grade adenoma in an Apcmin mouse colon cancer model and suppressed the expression of WNT target genes in human colorectal cancer cells through epigenetic modification recruiting histone methyltransferase SET domain bifurcated 1 (SETDB1). Extensive microarray analysis of human colorectal cancers also showed a negative correlation between the Notch1 target gene, Notch-regulated ankyrin repeat protein 1 (NRARP), and WNT target genes. Notch is known to be a strong promoter of tumor initiation, but here we uncovered an unexpected suppressive role of Notch1 on WNT/β-catenin target genes involved in colorectal cancer.

Introduction

Activation of the adenomatous polyposis coli/β-catenin (APC/β-catenin) pathway is a crucial initiating event in human colorectal cancer (CRC) (1, 2). The multistage progression of CRC is then followed by sequential activation of oncogenes and inactivation of tumor-suppressor genes, including K-RAS, TGFβ/SMAD4, and TP53 (2, 3). Although the implications of these genetic alterations are well characterized in the adenoma-carcinoma sequence, limited studies have been conducted to examine the influence of other molecular signaling pathways on these tumors.

Signaling pathways, such as wingless-type MMTV integration site (WNT), BMP, Hedgehog, and Notch, are important not only in embryonic development, but also in adult intestinal homeostasis (4, 5). In the intestine, WNT signaling is crucial for the proliferation and maintenance of intestinal stem cells and progenitor cells (6). Notch signaling regulates cell fate decisions between secretory and absorptive cell lineages (7, 8). The Notch signaling pathway also plays a role in the maintenance of proliferating progenitors (9, 10). Under normal conditions, these 2 pathways are exclusively required for maintaining intestinal stem cells (7). Activation of the WNT/β-catenin signaling pathway is the main rate-limiting step of CRC initiation (1), and Notch signaling has been shown to promote CRC initiation in a mouse Apcmin tumor model (11, 12). Therefore, the Notch and WNT pathways may function cooperatively in intestinal epithelium and tumors.

γ-Secretase inhibitor has been utilized to prevent intestinal tumor growth in Apcmin mice (10), which showed reduced proliferation of tumor cells and increased goblet cell conversion following γ-secretase inhibitor treatment. In contrast, overexpression of the Notch intracellular domain facilitates tumor proliferation of tumor cells and increased goblet cell conversion following γ-secretase inhibitor treatment. In contrast, overexpression of the Notch intracellular domain facilitates tumor proliferation of tumor cells and increased goblet cell conversion following γ-secretase inhibitor treatment. In contrast, overexpression of the Notch intracellular domain facilitates tumor proliferation of tumor cells and increased goblet cell conversion following γ-secretase inhibitor treatment. In contrast, overexpression of the Notch intracellular domain facilitates tumor proliferation of tumor cells and increased goblet cell conversion following γ-secretase inhibitor treatment.

We investigated the role of Notch signaling in established CRC tumors. We did not examine this signaling during the initial phase of transformation because it has been clearly shown in many studies that Notch signaling promotes the onset of intestinal tumors (11). Thus, after tumor onset, we reexamined the characteristics of Notch-activated tumors in RosaN1/Ros/N/Apcmin mice in which tumor initiation had progressed compared with Apcmin mice (11). Unexpectedly, these tumors showed low-grade adenoma features, including columnar epithelial morphology and a restoration of the adherens junction. Concomitant microarray analysis demonstrated Notch-mediated downregulation of Tcf4/β-catenin target genes, even in the absence of functional Apc. Microarray analysis of human CRC patient data further reinforced the negative correlation between Notch and WNT signaling activity in CRC. Our study examining the effect of Notch signaling in CRC cell lines (CRCLs) revealed that Notch signaling leads to a repressive...
Results

Histological characterization of Notch-activated Apcmin tumor after its onset. To examine how Notch activation affects intestinal tumors, we generated an intestinal gain-of-Notch model by crossing Apcmin background mice with Rosa-N1icd (RN1) mice, which have a transgene composed of a floxed Neo/STOP cassette followed by N1ICD without the PEST domain in the Rosa26 locus (15) and the Villin-Cre (Vil-Cre) transgene (16). These mice contain Notch-activated intestinal epithelium in which intestinal tumors occur upon the loss of heterogeneity of the Apcmin locus. A detailed description of these mice is provided in Supplemental Information and in Supplemental Figure 1 (supplemental material available online with this article; doi:10.1172/JCI61216DS1).

Consistent with the results of a previous study (11), 7-week-old Vil-Cre;RosaN1+/RN1;Apcmin mice developed several tumors in their small and large intestines, while tumors were not or were rarely detected in control Apcmin mice. Since the onset of tumor formation was clearly accelerated in Vil-Cre;RosaN1+/RN1;Apcmin mice compared with control Apcmin mice in our study as well as in a previous study (11), we collected tumor tissues from 7-week-old Vil-Cre;RosaN1+/RN1;Apcmin mice and 10- to 15-week-old Apcmin mice. For histological analysis, we used tumors of similar size from each genotype for comparison.

Interestingly, a detailed histological analysis of tumors in Vil-Cre;RosaN1+/RN1;Apcmin mice, particularly those in the colon, showed low-grade adenoma characteristics with enhanced epithelial cell morphology, while control tumors from Apcmin mice showed high-grade adenoma characteristics (Figure 1, A and B). Tumors from Vil-Cre;RosaN1+/RN1;Apcmin mice did not form tightly packed cell agglomerations as observed in Apcmin tumors. Instead, they formed an extended single layer of epithelial cells. Low-grade adenoma characteristics were also observed in older (10- to 15-week-old) moribund Vil-Cre;RosaN1+/RN1;Apcmin mice (not shown), suggesting that mortality of Vil-Cre;RosaN1+/RN1;Apcmin mice was not due to further progression of tumor severity.

Next, we investigated the localization of β-catenin. Control Apcmin tumors showed prominent nuclear and cytoplasmic staining of β-catenin, a hallmark of active WNT signaling (Figure 1, C and D). Although nuclear β-catenin was still detectable in Notch-activated Apcmin tumors, most of the β-catenin was present in the plasma membrane (Figure 1, F and G). Importantly, the expression level of E-cadherin was restored in the plasma membrane of Notch-activated Apcmin tumors (Figure 1, E and H). Immunostaining with E-cadherin and α-catenin showed that while adherens junctions were disrupted in Apcmin tumors (Figure 1, I), E-cadherin and α-catenin were colocalized in the plasma membrane of Notch-activated Apcmin tumors (Figure 1, J). Moreover, double staining of E-cadherin and ZO-1, a marker for tight junctions, clearly showed that lateral and apical membranes were well established in Notch-activated Apcmin tumors (Figure 1, K and L). These results show that Notch-activated Apcmin tumors display low-grade adenoma with restored epithelial characteristics.

Downregulated WNT target genes in Notch-activated Apcmin tumors. Milder histological characteristics of Notch-activated Apcmin tumors compared with those of normal Apcmin tumors prompted us to further analyze molecular changes in these tumor models. Because tumor onset was accelerated in the Vil-Cre;RosaN1+/RN1; Apcmin mice as described above, we collected tumors of similar
size from 7-week-old Vil-Cre;RosaN1+/RN1;Apcmin mice and 10-week-old Apcmin mice. First, we determined the changes in expression between tumors from these 2 genotypes and then compared the entire signature to changes between normal intestinal epithelium and Apcmin tumors (Figure 2A and Suppl. Table 1). Among 2,750 genes significantly altered during Apcmin tumorigenesis (normal tissue vs. tumor), activation of Notch signaling suppressed approximately 40% of tumor-associated genes toward normal levels (Figure 2A). In cluster A, depicted in the figure, we identified genes related to cell metabolism and differentiation using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, confirming the histological observation that Notch signaling promotes epithelial differentiation of intestinal tumor cells (Suppl. Table 2).

Strikingly, the KEGG pathway analysis of cluster B showed that WNT signaling pathway genes were downregulated in Notch-activated Apcmin tumors (Figure 2, F and G). Importantly, we also observed that PROX1, a β-catenin target gene known to promote intestinal tumor progression (21), was nearly absent in Notch-activated Apcmin tumors (Figure 2G). These data show that Notch signaling specifically counteracts deregulation of approximately 40% of Apcmin tumor-associated genes by modulating factors downstream of the WNT signaling pathway.

**Figure 2**
Decreased levels of WNT target genes in Notch-activated Apcmin tumors. Colons (normal) from 10-week-old WT and 7-week-old Vil-Cre;RosaN1+/RN1 (N1) mice and colonic tumor tissues (tumor) from 10-week-old Apcmin (APC) and 7-week-old Vil-Cre;RosaN1+/RN1;Apcmin mice were used for gene expression and histological analysis. (A) Heat map of gene expression levels. Clusters A and B indicate genes that reverted to levels in normal tissues upon Notch activation in the tumors. (B–E) Real-time qRT-PCR analysis (B–E). Note the reduced expression levels of WNT target genes (Axin2, Wif1, Nkd1, and Apcdd1) in APC:N1 tumors compared with those of APC tumors. Bars indicate mean + SD. *P < 0.05; **P < 0.01; ***P < 0.001. (F and G) In situ hybridization (F) and fluorescent immunostaining (G) of Apcmin (upper panels) and Vil-Cre;RosaN1+/RN1;Apcmin (lower panels) tumors. Note that the expression levels of Axin2, Wif1, Apcdd1, and PROX1 were reduced or absent in Vil-Cre;RosaN1+/RN1;Apcmin tumors. Dotted lines indicate tumor regions. Scale bars: 100 μm (F); 50 μm (G).
our mouse models as reported (22–24). To confirm the correlation between Nrarp expression and Notch signaling activity, we used transgenic Notch reporter (TNR) mice, which have a Notch-responsive GFP reporter that faithfully recapitulates endogenous Notch activity (25). In Apc<sup>min</sup>;TNR mice, expression levels of GFP and Nrarp were reduced to approximately half in the tumor compared with nontumor intestinal epithelial tissues, while the expression level of Hes1 slightly increased (Figure 3A). Consistently, in situ hybridization and real-time qRT-PCR analysis showed that the Nrarp expression level was increased in Notch-activated Apc<sup>min</sup> tumors compared with normal Apc<sup>min</sup> tumors (Figure 3, B and C). These data show that Nrarp reports Notch signaling activity more accurately than Hes1 in intestinal tumors.

Using Nrarp as an indicator of Notch signaling activity in CRC, we used both Pearson’s and Spearman’s correlation analysis (P < 0.05; see Methods) to identify genes correlated with Nrarp in 2 independent human microarray data sets (GEO GSE5206 and GSE2109). These data sets contained a sufficient amount of human patient data to perform our statistical analysis. Coexpression analysis provided a set of genes whose expression was positively or negatively correlated with Nrarp (human Nrarp CO-DEG, where DEG indicates differentially expressed gene) expression. If the Nrarp expression level accurately predicts Notch activity in human CRC, genes that are filtered using this coexpression analysis would be similar to genes identified by comparing control and Notch-activated Apc<sup>min</sup> tumors. We analyzed genes that commonly occur in mouse Notch DEG sets between control and Notch-activated Apc<sup>min</sup> tumors (Supplemental Table 1; P < 0.05, 2-fold) and human Nrarp CO-DEG (genes that correlate with Nrarp; P ≤ 0.05) (Supplemental Table 4; 239 genes coregulated in mouse Notch-DEG and human Nrarp CO-DEG). The heat map of these genes, sorted based on the expression level of Nrarp, showed an 85.8% identical expression change between mouse and human (Figure 4A), suggesting that Nrarp is a reliable marker for Notch signaling activity in human CRC. Genes associated with the differentiation of intestinal epithelium, CDX1 and CDX2, showed higher expression levels in Nrarp<sup>hi</sup> human colonic tumors (Figure 4B and ref. 26). VIMENTIN (27) and PROX1, which are associated with tumorigenic activities such as EMT and tumor progression, exhibited lower levels of expression in Nrarp<sup>hi</sup> human colonic tumors (Figure 4B). In particular, a negative correlation between Nrarp and PROX1, a known WNT target gene important in CRC progression, further supports our previous observation that Notch signaling can attenuate WNT/β-catenin activity under destruction complex–deregulated conditions.

Next, to investigate Notch signaling activity during CRC development in humans, we compared Nrarp expression levels among 3 human microarray data sets. Nrarp expression was significantly decreased along with human CRC progression (Figure 4C; GEO GSE5206, normal to adenoma, adenocarcinoma). Although the stepwise differences were minor, the overall pattern showed a significant decrease in expression (Figure 4C; P = 0.0002 using 1-way ANOVA). Data sets (GEO GSE4107 and GSE8671) were used to confirm the reduction of Nrarp expression in carcinomas compared with that of normal intestinal epithelium (Figure 4C; P = 0.0012 and P < 0.0001, respectively). Recently, Smith et al. reported microarray data coupled with patient survival information (GEO GSE17538) (28). We divided this patient data into 2 groups, Nrarp<sup>hi</sup> (upper 25%) and Nrarp<sup>lo/–</sup>, and constructed a Kaplan–Meier plot (Figure 4D). Interestingly, significantly longer and higher survival was observed for patients with high Nrarp expression, suggesting that Nrarp expression is a strong predictor of patient survival (P = 0.022). Based on Nrarp expression as a surrogate marker, we show that Notch activity in CRCLs decreases during CRC progression.

**Notch signaling suppresses the expression of WNT target genes in human CRC cells.** To further investigate the crosstalk between Notch and WNT signaling, we sorted Nrarp in human CRCLs based on Nrarp expression levels. Interestingly, Nrarp<sup>hi</sup> CRCLs highly expressed CDX1 and CDX2, but did not express or only minimally expressed VIMENTIN and PROX1 (Figure 5A). In contrast, Nrarp<sup>lo/–</sup> CRCLs highly expressed VIMENTIN and PROX1, but did not express or only minimally expressed CDX1 and CDX2 (Figure 5A). In order to determine whether Notch signaling is really active in the Nrarp<sup>hi</sup> CRCLs, but not in Nrarp<sup>lo/–</sup> CRCLs, we examined the expression of cleaved Notch1. As expected, cleaved Notch1 was readily detected in the Nrarp<sup>hi</sup> CRCLs, such as SNU61 and LOVO, but not in Nrarp<sup>lo/–</sup> CRCLs, such as SW620 and COLO205 (Figure 5B). In order to investigate whether the NRARP expression is directly induced by Notch signaling, we tested the effect of cycloheximide, a new protein synthesis inhibitor, on Notch signaling-induced NRARP expression in SNU61 and LOVO cells. The decrease of NRARP expression by γ-secretase inhibitor (DAPT) treatment in SNU61 and LOVO cells was reversed by washout of DAPT in the absence
or presence of cycloheximide (Figure 5C), indicating that the induction of NRARP by Notch activation in the Nrarp hi CRCLs does not require de novo protein synthesis. In accordance with the previous reports (22–24), our data show that NRARP is a direct target of Notch signaling in the Nrarphp CRCLs.

To determine whether the characteristics of CRCLs could be affected by Notch activity, Nrarphp CRCLs were treated with DAPT. Surprisingly, CDX1 and CDX2 expression levels in Nrarphp CRCLs, such as LOVO and SNU61, decreased following treatment with DAPT, whereas VIMENTIN expression increased (Figure 5D). In contrast, when the Nrarpmid/lo CRCLs SW620 and COLO205 were transfected with constitutively active Notch1 (ΔEN1), expression levels of CDX1 and CDX2 increased, while VIMENTIN expression decreased (Figure 5D). Taken together, these results suggest that Notch activity affects the characteristics of CRCLs.

Since Notch signaling downregulates the WNT target genes Axin2, Wif1, Nkd1, and Apedd1 in our mouse models (Figure 2, B–G), we further investigated whether Notch signaling also affects the expression of WNT target genes in human CRCLs. We determined whether inhibition of Notch signaling results in the expression of WNT target genes in the Nrarp hi CRCLs.

Figure 4
Conserved Notch signatures in both mouse and human intestinal tumors. (A) Heat map comparison between Notch-responsive genes (clusters A and B in Figure 3A) from mouse models and microarray data sets from human CRC patients (GEO GSE5206 and GSE2109). Note that genes upregulated upon Notch activation in Apcmin tumors have higher expression levels in Nrarphp tumors compared with those of Nrarplo tumors and vice versa. Asterisk indicates the group of genes that do not correlate with mouse and human data. APC, Vil-Cre;Apcmin. (B) Positive correlation of CDX1 and CDX2 (transcription factor for epithelial differentiation) and negative correlation of VIMENTIN (epithelial-mesenchymal transition marker) and PROX1 (tumor progression related gene) with Nrarp, respectively. \( \rho \), correlation coefficient. Statistics are by Pearson’s correlation. (C) Expression levels of Nrarp in the 3 independent microarray data sets (GEO GSE5206, GSE4107, and GSE 8671). AD, adenoma; AC, adenocarcinoma; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; C, cancer. Note the reduced Nrarp expression in CRCs compared with normal tissue. \( **P < 0.001; ***P < 0.005 \). Statistics for far-left graph are by ANOVA. (D) Positive correlation between Nrarp expression and patient survival from microarray data set (GEO GSE17538). The Kaplan-Meier method was used to estimate survival of the 2 groups; Nrarphp (solid line) and Nrarpmid/lo (dotted line).
Figure 5
Notch signaling promotes differentiation of CRCLs. (A) Real-time qRT-PCR analysis of Nrarp, CDX1, CDX2, VIMENTIN, and PROX1 in various human CRCLs. Note that Nrarp<sup>high</sup> CRCLs showed high expression levels of CDX1 and CDX2, while Nrarp<sup>low</sup> CRCLs highly expressed VIMENTIN and PROX1. (B) Western blot analysis of NICD and NRARP in CRCLs. Note that the expression of cleaved Notch 1 was well correlated with the expression levels of NRARP in SNU61, LOVO, SW620, and COLO205 cells. (C) Real-time qRT-PCR analysis of Nrarp expression in cycloheximide-treated CRCLs. LOVO and SNU61 cells were treated with DAPT (1 µM) for 48 hours to block Notch-signaling activity. Cells were then washed, and medium containing DAPT (mock washout) or medium lacking DAPT (washout) with or without 20 µM cycloheximide was added. After 12 hours of additional culture, cells were harvested, and Nrarp RNA levels were determined. (D) Real-time qRT-PCR analysis of CDX1, CDX2, and VIMENTIN in CRCLs. SNU1040, LOVO and SNU61 cells were treated daily with vehicle or 1 µM DAPT. SW620 and COLO205 cells were transfected with 2 µg of mock vector or ΔEN1, a constitutively active form of Notch1. Levels of CDX1, CDX2, and VIMENTIN transcripts were measured after 48 hours. Bars indicate mean ± SD. *P < 0.05; **P < 0.001.
We also determined whether activation of Notch signaling in Nrarp^+/- CRCCLs leads to inhibition of WNT target gene expression. Expression levels of WNT/β-catenin target genes, including PROX1, AXIN2, c-MYC, and APCDD1, were decreased by ΔEN1 in SW620 and COLO205 CRCCLs (Figure 7, A and B), while MMP-2 expression did not change, suggesting that Notch signaling suppresses WNT target genes.

We next investigated whether Notch signaling influences tumor growth. As expected, the treatment of DAPT in the Nrarp^+ SNU61 and LOVO CRCCLs increased the proliferation of these cells, although the increase was not dramatic (Supplemental Figure 2A). Consistently, when ΔEN1 was expressed on the Nrarp^+- COLO205 and SW620 CRCCLs, it significantly inhibited the proliferation of these CLCs (Supplemental Figure 2B). Taking these data together, we concluded that Notch signaling suppresses the expression of WNT target genes in human CRC cells.

Notch signaling suppresses the expression of WNT target genes by modifying epigenetic status. Since Notch signaling suppresses WNT/β-catenin activity under destruction complex–deregulated conditions, we speculated that Notch signaling could modulate the transcriptional activity of WNT target genes. To examine this possibility, we tested the binding activity of β-catenin/TCF4 to WNT/β-catenin target promoter regions. Interestingly, both DAPT treatment and DN-MAML overexpression in LOVO cells resulted in increased binding activity of β-catenin/TCF4 to the PROX1, Axin2, and c-MYC promoter regions (Figure 8A and Supplemental Figure 3), suggesting that Notch activation affects the binding affinity of β-catenin/TCF4 to target gene promoters.

We next determined whether the binding affinity of β-catenin/TCF4 to the promoter is affected by Notch signaling through epigenetic modification. Deacetylation and methylation of histones play an important role in transcription regulation. Specifically, trimethylation of the histone H3 tail at lysine-9 (H3K9me3) and lysine-27 (H3K27me3) is associated with gene silencing, while trimethylation of the histone H3 tail at lysine-4 (H3K4me3) is associated with gene activation (29–31). The levels of H3K9me3 and H3K27me3 apparently decreased following DAPT treatment and overexpression of DN-MAML in LOVO cells, while H3K4me3, a marker of active gene transcription, increased (Figure 8A and Supplemental Figure 3). In contrast, the levels of H3K9me3 and H3K27me3 in Nrarp^+- SW620 CRCCLs apparently increased by ΔEN1 in WNT/β-catenin target promoter regions (Figure 8B and Supplemental Figure 4), while H3K4me3 and H3K4ac, markers of active genes, did not change (data not shown). These results show that Notch signaling can inhibit the expression of WNT/β-catenin target genes through epigenetic modification in CRC.

We further investigated how Notch signaling negatively regulates WNT target gene expression through histone modification.
NLK, known to antagonize WNT signaling, acts downstream of the Notch pathway to inhibit TCF/β-catenin signaling during mesoderm induction in sea urchin embryos (32). NLK also phosphorylates SETDB1, a histone methyltransferase, leading to the formation of a corepressor complex that inactivates the activity of the transcriptional factor PPARγ through histone 3-K9 methylation (33). This suggests that NLK and SETDB1 affect Notch-mediated inhibition of WNT target gene expression by modifying histone status. To examine this possibility, we transfected Nrarplo−/− SW620 cells with NLK siRNA and SETDB1 siRNA (Supplemental Figure 5). These cells were then retransfected with control and ΔEN1. Intriguingly, downregulation of the WNT target genes PROX1, c-MYC, and Axin2 by Notch signaling was significantly abolished by both NLK siRNA (Figure 8C) and SETDB1 siRNA (Figure 8D) treatment, suggesting that NLK and SETDB1 are required for downregulation of WNT target genes by Notch signaling.

To investigate whether Notch signaling affects the recruitment of NLK and SETDB1 in WNT/β-catenin target promoter regions, we performed a ChIP assay using Nrarplo−/− SW620 cells transfected with ΔEN1. As shown in Figure 8E, NLK and SETDB1 were readily recruited to WNT/β-catenin target promoter regions by ΔEN1. These results demonstrate that NLK and SETDB1 are involved in the epigenetic regulation of gene expression by Notch signaling.

Finally, we investigated the effects of DAPT in NRARPlo−/− CRCLs. As expected, we could not detect any significant differences in NRARPlo−/− CRCLs after DAPT treatment. Thus, we concluded that the DAPT treatment in the NRARPlo−/− cell lines did not further affect the epigenetic status and characteristics of these cells. A detailed Description is provided in Supplemental Information and Supplemental Figure 6.

Discussion

In the intestine, Notch signaling is a crucial signaling component for the maintenance of intestinal progenitors and stem cells and for the regulation of binary cell fate decisions (7, 8). Notch activation also significantly accelerates tumor formation in terms of number and onset timing (11). Since this signaling pathway has been shown to positively regulate the proliferation of intestinal progenitors and the initiation of tumor formation, this pathway is defined as oncogenic. Generally, understanding the oncogenic role of specific signaling components is important for identifying therapeutic targets of cancers. Consequently, γ-secretase inhibitors, which were originally developed to treat Alzheimer patients, are also therapeutic candidates for the treatment of CRCs. However, our study shows that the role of Notch signaling in CRCs is complex. An incomplete understanding of the role of this pathway in CRC will prevent the successful development of treatment methods. Here, we discuss a new role for Notch in CRCs and the significance of this finding.

Dual roles of Notch signaling in intestinal tumorigenesis. Notch signaling is generally known to be oncogenic in various tissues, although the loss of this signaling can also result in tumor formation (34, 35). In the intestine, Notch signaling was thought to exhibit oncogenic potential by regulating the proliferation of intestinal progenitors (9, 36). In accordance with these reports, activation of Notch signaling strongly prompts tumorigenic activity in Apcmin backgrounds (11, 12). We also observed that Vil-Cre;RosaN1+/RN1;Apcmin mice developed numerous tumors in the small and large intestines at an early age, and the onset of tumor formation was accelerated compared with that of control Apcmin mice. Our results are consistent with a recent report stating that Notch and WNT signals cooperate to cause increased proliferation of intestinal progenitors did not affect the proliferation of colon cancer cells.
in culture or in grafting experiments. These results are inconsistent with those of previous studies, demonstrating that activated Notch signaling facilitates tumor formation in Apcmin mice through increased proliferation of tumor cells (10, 11). Other studies have also reported that Aes can modulate other signaling pathways, such as WNT, TGF-β, and Hedgehog, which have critical roles in CRCs (40–42). Therefore, further characterization to determine whether Aes affects CRCs through Notch or other signaling pathways is required. Previously, Babaei-Jadidi et al. reported that the loss of Fbxw7 in Ape<sup>mm</sup> mice resulted in early tumor initiation compared with Ape<sup>mm</sup> mice (43). However, genetic deletion of Fbxw7 in the intestine resulted in multiple deregulations of other molecules, such as Jun and DEK, as well as in Notch signaling. Fbxw7 mutant tumors showed accumulation of the DEK protooncogene. Thus, it is not clear whether accelerated tumor initiation in Fbxw7 in Ape<sup>mm</sup> mice is due to activated Notch1.

In the present study, we directly activated Notch signaling in Ape<sup>mm</sup> mice using the RosaN1<sup>ΔEN1</sup> knockin mouse, with which we could activate components downstream of Notch and rescue or reverse the loss-of-Notch mutant phenotype (44). Histological
analysis of Vil-Cre;RosaN1<sup>−/−</sup>;Apc<sup>min</sup> mice revealed that Notch-activated tumors in these mice exhibit low-grade adenoma characteristics despite an initial increase in tumor formation. Additionally, Notch activation can revert approximately 40% of gene expression changes associated with Apc<sup>min</sup> tumors. The most striking finding was that WNT/β-catenin target gene expression was downregulated upon activation of Notch in Apc<sup>min</sup> tumors. This was also demonstrated in human CRCs and CRCLs. Furthermore, we found that Notch signaling regulates the expression of WNT/β-catenin target genes through epigenetic modification in CRC cells. Based on this observation, our present study demonstrates that Notch signaling acts negatively on CRC progression through downregulation of WNT/β-catenin target genes, which explains how Notch-activated Apc<sup>min</sup> tumor remains in a low-grade state.

Crosstalk between Notch and WNT signaling in intestinal tumorgenesis and Nrarp, a reliable marker for Notch signaling activity. Several genetic observations suggest that functional crosstalk exists between WNT and Notch signaling (45). Initial insights on how Notch signaling represses WNT signaling arose from studies conducted using <i>Drosophila</i>, in which a direct physical interaction between NICD and Dishevelled has been suggested as mediating the mutual inhibition of these 2 signaling pathways (46). In the skin, Notch1 activation inhibits the WNT signaling pathway by downregulating WNT ligand gene expression, which is mediated by p21 transcription (47). Additionally, GSK3β has been suggested to be a crucial link between these 2 signaling pathways (44, 48, 49). However, downregulation of WNT/β-catenin target genes in Apc<sup>min</sup> tumors by active Notch signaling is not easily explained by such mechanisms because they are upstream regulators of the APC/β-catenin complex. Here, we observed molecular crosstalk between WNT and Notch signaling, which takes place downstream of the APC/β-catenin complex; Notch signaling can suppress the transcriptional activity of WNT target genes by modulating their histone status. Additionally, the negative correlation between Notch and WNT activity was observed in our animal model as well as in human CRC patient arrays and human CRCLs.

Recently, Alves-Guerra et al. reported that MAML1 is a coactivator of β-catenin activity and that the C-terminal region of MAML1 (aa 640–840) is critical to β-catenin activity (50). In our study, we used DN-MAML (aa 13–74) to block Notch activity by binding to NICD. When DN-MAML (aa 13–74) was transiently expressed in various NRARP<sup>β</sup> colon cancer cells, it did not affect β-catenin activity, while Notch activity was effectively blocked. Our data show that the N-terminal domain of MAML1 is not required for β-catenin activity. Alves-Guerra et al. also demonstrated that the increase in transcriptional activity of the β-catenin pathway is due to MAML in addition to crosstalk with the Notch signaling pathway. However, we observed that γ-secretase inhibitor (DAPT) treatment of NRARP<sup>β</sup> colon cancer cells inhibited Notch activity similarly to DN-MAML (aa 13–74), suggesting that the expression of WNT/β-catenin target genes is regulated in a Notch signaling–dependent manner in NRARP<sup>β</sup> CRCLs.

The best characterized targets of Notch-mediated activation include members of the hairy and enhancer of split (HES) and HES-related repressor protein (HERP) families of basic helix-loop-helix (bHLH) transcriptional repressors (51). Although <i>Hes1</i> is a well-known Notch target gene, it is also regulated by Notch-independent signaling pathways, such as the WNT pathway. Recently, Peignon et al. reported that <i>Hes1</i> was induced directly by β-catenin signaling via the conserved Tcf-binding site of the <i>Hes1</i> promoter region (14). Indeed, <i>Hes1</i> expression is elevated in Apc<sup>min</sup> tumors and human CRCs (10). Moreover, <i>Hes1</i> expression is not decreased in Apc and Rbp double-knockout mice compared with Apc-null mice (14). In this study, we identified Nrarp as a reliable Notch target gene for representing the endogenous level of Notch signaling activity in intestinal tumors. Nrarp is a Notch target gene for feedback inhibition of its transcriptional activity. Notch signaling activity influences the expression of Nrarp as it does other feedback inhibitors such as Axin2 (WNT/β-catenin signaling) and Sox3 (Jak/Stat3 signaling) (17). The expression level of Nrarp is significantly reduced in Notch1<sup>−/−</sup> embryos (24). Moreover, the expression of Nrarp, but not <i>Hes1</i>, was well correlated with the expresional changes of a TNR (TNR-GFP) (Figure 3A). These data show that Nrarp is a reliable sensitivity and dependency marker for Notch signaling activity.

In conclusion, we demonstrate the dual roles of Notch signaling in intestinal tumor progression; Notch both suppresses tumor progression and enhances tumor initiation. In particular, Notch signaling has a negative effect on CRC progression by downregulating WNT/β-catenin target genes. Thus, the results of our study will help further the understanding of the dual roles of Notch signaling in intestinal tumorgenesis and provide critical clues for improving treatments for colonic neoplasia and cancers. We are only beginning to understand the complex interplay between these signaling pathways, and further studies will reveal more detailed mechanisms by which crosstalk contributes to intestinal tumor progression. This will help us to unveil a promising tool for restoring the deregulated WNT pathway in CRCs.

Methods
Mice. The Apc<sup>min</sup> mouse line and Vilino-Cre (Vil-Cre) transgenic mouse line were purchased from The Jackson Laboratories and maintained in our animal colony under our institutional guidelines. Rosa-Notch1 mice (a gift from D. Melton, Harvard Stem Cell Institute, Harvard University, Boston, Massachusetts, USA) harbor the intracellular domain of mouse Notch1 in the ubiquitously expressed Rosa26 locus, the expression of which is blocked in the absence of Cre. Rosa-Notch1 mice were crossed with the Vil-Cre mice and Apc<sup>min</sup> mice to generate Vil-Cre;RosaN1<sup>−/−</sup>;Apc<sup>min</sup>. The TNR mice were a gift from N. Gaiano (Johns Hopkins University, Institute for Cell Engineering, School of Medicine, Baltimore, Maryland, USA).

Cell culture. Human CRC cells were obtained from ATCC and the Korean Cell Line Bank (52). Cells were maintained in RPMI 1640 medium (100 U/ml penicillin; 100 µg/ml streptomycin) supplemented with 10% FBS (HyClone Labs) in an atmosphere of 95% air and 5% CO2 at 37°C. All experiments were performed with cells at 50%–70% confluence.

Tissue preparation, immunohistochemistry, and in situ hybridization. The intestinal tract was flushed gently with cold PBS, followed by a flush with 4% paraformaldehyde in PBS. For histological analyses, tissues were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin wax for sectioning. Sections (3–4 µm) were stained using H&E. For immunohistochemistry, paraffin-embedded sections were rehydrated, and antigenic epitopes were exposed using boiling citrate buffer or Tris/EDTA buffer. Sections were incubated in blocking solution (3% BSA, 5% goat serum or horse serum, and 0.5% Tween 20 in PBS) at room temperature (RT) for 2 hours, followed by an additional incubation with Abs to β-catenin (1:200, BD Biosciences; 1:200, Santa Cruz Biotechnology Inc.), E-cadherin (1:200, BD Biosciences), α-catenin (1:50, Santa Cruz Biotechnology Inc.), ZO-1 (1:200, Zymed), Prox1 (1:100, Millipore), Axin2 (1:200, Abcam), and Apccdh1 (1:200, Abcam). Specific binding was detected using an Envision kit (DAKO) or Alexa Fluor 488-labeled (green) and/or Alexa 3257
Fluor 594–labeled (red) Abs (Molecular Probes). For in situ hybridization, tissues were fixed in 10% formalin overnight at RT and embedded in paraffin wax for sectioning. Dig-labeled antisense RNA transcripts were used for hybridization and visualized using anti-Dig-alkaline phosphatase Ab (BM) and BM purple reagent (BM).

**Microarray analysis and statistics.** For mouse tissue mRNA analysis, total RNA was isolated using Trizol (Invitrogen) and purified using RNeasy columns (Qiagen). The RNA was reversed transcribed, amplified, and hybridized onto Sentrix Mouse-6 v1 BeadChips (Illumina) according to the manufacturer’s instructions. Probe intensity was normalized using the quantile method (53) at the log2 scale. To identify tumor DEGs (T-DEG), the value, which indicates the significance of correlation of a gene with $Nairp$ between $Nairp$ and other genes in each data set (GEO GSE2109 and GSE5206). We then calculated the $P$ values for each type of correlation using null correlations between $Nairp$ and randomly permuted genes. Random permutation experiments were repeated 100 times. For each gene in the data set, 2 $P$ values from Pearson’s and Spearman’s correlations were integrated into a combined $P$ value by using the Liptak-Stouffer Z-method (54). Finally, 2 combined $P$ values from 2 data sets were recombined to generate an overall $P$ value, which indicates the significance of correlation of a gene with $Nairp$ in both data sets. Human NRARP CO-DEG was then identified as a gene with an overall $P$ value of less than 0.05. KEGG pathways significantly associated with a set of genes were identified using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (55).

**Retrolateral expression vectors and siRNAs.** The ΔEN1 cDNA was cloned into the Hpv1 site of pMSCV. ΔEN1 is a constitutively active form of Notch1 that lacks the extracellular domain. This form of Notch1 can bypass S2 cleavage and is readily processed by γ-secretase in a ligand-independent manner, but can still be blocked by a γ-secretase inhibitor. The MigR1-DN-MAML (dominant-negative form of MAML1) was a gift of J. Aster (Brigham and Women’s Hospital, Boston, Massachusetts, USA). siRNA to NLK and SETDB1 was designed and synthesized by Dharmacon (Thermo Scientific).

**Western blot and RT-PCR analyses.** For Western blot analysis, equal amounts of whole-cell extracts or tissue extracts were separated using SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with Abs to Mib1 (gift from P. Gallagher, Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana, USA), cleaved Notch1 (Abcam), Hes1 (gift from T. Sudo, Pharmaceutical Research Laboratories, Toray Industries Inc., Tekiro, Kamakura, Japan), Math1 (Abcam), NRARP (BD Biosciences – Clontech), Prox1 (Chemicon), Axin2 (Abcam), C-myc (Santa Cruz Biotechnology Inc.), APCDD1 (Abcam), MMP2 (Cell Signaling Technology), and β-actin (Sigma-Aldrich). Protein bands were detected by enhanced chemiluminescence (Amersham Biosciences). For RT-PCR and real-time qRT-PCR analysis, total RNA was isolated from freshly dissected intestines using Trizol reagent (Life Technologies), and complementary DNA synthesis was performed according to the manufacturer’s instructions (Omniscript Kit; Qiagen). PCR quantification was conducted using the SYBR green method. Primer information is available in Supplemental Data.

**ChIP analysis.** ChIP analyses were performed using the EZ-Chip kit according to the manufacturer’s protocol (Upstate Biotechnology). Specific PCR primers were designed to contain putative β-catenin/TCF4–binding sites as determined by TFSEARCH, version 1.3, and MatInspector, version 3.0, Genomax Software. Immunoprecipitation was performed using Abs to β-catenin (Santa Cruz Biotechnology Inc.), TCF-4 (Santa Cruz Biotechnology Inc.), H3K27me3 (Abcam), H3K9me3 (Abcam), H3K4me3 (Abcam), NLRK (Abcam), and SETDB1 (Abcam). Immunoprecipitated complexes were isolated and 1 μl of immunoprecipitated purified DNA was amplified using pairs of primers that cover the WNT/β-catenin–binding sites in promoter regions of WNT target genes. Exon 6 of GAPDH served as a negative control. Primer information is available in the Supplemental Data.

**Statistics.** All values are given as mean ± SD. Statistical comparisons were made by 2-tailed Student’s t test. A $P$ value of less than 0.05 was considered to be statistically significant.

**Study approval.** All mouse lines were maintained in specific pathogen-free conditions at the Institute of Laboratory Animal Resources, Seoul National University. All animal experiments were approved by the ethical committees at Seoul National University (permit number SNU-081001-9).

**Acknowledgments**

This work was supported by grants from the Basic Science Research Program through the National Research Foundation of Korea (2012-0000121), a Global Frontier Project grant (NRF-M1AAXA002-2011-0028413) of the National Research Foundation (NRF) funded by the Ministry of Education, Science and Technology of Korea, the Bio and Medical Technology Development Program of the NRF funded by the Korean government (MEST) (2011-0019269), and the National R&D Program for Cancer Control, Ministry of Health and Welfare, the Republic of Korea (0920310).

Received for publication September 28, 2011, and accepted in revised form July 5, 2012.

Address correspondence to: Young-Yun Kong, Department of Biological Sciences, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul, 151-747, Republic of Korea. Phone: 82.2.880.2638; Fax: 82.2.872.1993; E-mail: ykong@snu.ac.kr.
research article

25. Duncan AW, et al. Integration of Notch and Wnt
24. Krebs LT, Deftos ML, Bevan MJ, Gridley T. The
22. Pirot P, van Grunsven LA, Marine JC, Huylebroeck
17. Joo EH, Zhang T, Domon C, Joo CK, Freund JN,
16. Costantini F. Wnt/beta-catenin/Tcf signaling
14. Ranganathan P, Weaver KL, Capobianco AJ.
12. Rottinger E, Croce J, Lhomond G, Besnardeau
10. Lachner M, O’Carroll D, Rea S, Mechtler K,
8. Braunstein E, Gumucio DL. Cis elements of the vil
7. Joo EH, Zhang T, Domon C, Joo CK, Freund JN,
6. Costantini F. Wnt/beta-catenin/Tcf signaling
4. Ranganathan P, Weaver KL, Capobianco AJ.
2. Pirot P, van Grunsven LA, Marine JC, Huylebroeck
1. Costantini F. Wnt/beta-catenin/Tcf signaling

The Journal of Clinical Investigation
http://www.jci.org
Volume 122 Number 9 September 2012

3259