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ORIGINAL ARTICLE

Heterogeneity of Jagged1 expression in human and mouse intestinal tumors: implications for targeting Notch signaling

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Inhibition of Notch signaling is effective in inhibiting colon tumorigenesis, but targeting specific components of the pathway may provide more effective strategies. Here we show that the expression of Jagged1, a ligand for canonical Notch signaling, was restricted to enteroendocrine cells or undetectable in the mucosa of the human small and large intestine, respectively. In contrast, increased expression characterized half of human colon tumors, although not all tumors with elevated Wnt signaling displayed elevated Jagged1. Increased Jagged1 was also present in intestinal tumors of $Apc^{1638N/+}$ and $Apc^{Min/+}$ mice, but to a higher level and more frequently in the former, and in 90% of mouse tumors Notch signaling was elevated when Jagged1 was elevated. In the human HT29Cl16E colonic carcinoma cell line, induction of goblet cell differentiation by contact inhibition of growth depended on the loss of Jagged1-mediated Notch activation, with signaling through Notch1 and Notch2 acting redundantly. Therefore, targeting of Jagged1 could be effective in downregulating Notch signaling in a subset of tumors, but may avoid the limiting gastrointestinal toxicity caused by pharmacological inhibition of Notch signaling.
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Introduction

The Notch signaling pathway is a key determinant of intestinal epithelial cell self-renewal and allocation of these cells to specific differentiation lineages (Milano *et al*., 2004; Fre *et al*., 2005; Stanger *et al*., 2005; van Es *et al*., 2005). In mammals, four Notch genes are expressed, each of which encodes a single-pass transmembrane receptor (Notch1–4). Each Notch receptor can be activated by cell-membrane-associated ligands belonging to the Jagged and Delta-like families. Upon ligand binding, Notch receptors are ultimately cleaved by a γ-secretase, which releases Notch intracellular domain (NICD) from the plasma membrane and initiates its subsequent translocation into the nucleus. Once there, NICD activates the expression of downstream target genes (Fryer *et al*., 2002) by binding to the conserved transcription factor, RBP-Jκ (Fortini and Artavanis-Tsakonas, 1994), among whose targets are the Hes (Hairy Enhancer of Split) basic helix–loop–helix transcriptional repressor family. In particular, NICD stimulates the expression of Hes1 (Jarriault *et al*., 1995), which represses the activity of Math1 (or Hath1, its human homologue) (Zheng *et al*., 2000; Yang *et al*., 2001). Inhibition of Notch signaling, thus, elevates Math1 expression, which, in the intestine, drives cells to differentiate along secretory cell pathways, prominently elevating the representation of goblet cells and hence mucin secretion (Jensen *et al*., 2000; Yang *et al*., 2001; van Es *et al*., 2005; Guilmeau *et al*., 2008).

Homeostasis of the intestinal mucosa is maintained through regulation of both proliferation and differentiation by Notch and Wnt signaling in cooperation with other intrinsic and extrinsic signals. Recent data have demonstrated that while the effects of Notch on intestinal cell proliferation are dependent on Wnt signaling, the effects on goblet cell differentiation are independent of Wnt (Fre *et al*., 2009). This emphasizes the importance of understanding the functional and regulatory interactions among Notch and Wnt signaling in intestinal cells and tumorigenesis. As regards regulatory interactions, it has been shown that a Notch ligand, Jagged1, is transcriptionally controlled by the β-catenin/TCF4 complex in the hair follicle of the inner ear (Estrach *et al*., 2006), and similar results were found by bioinformatic analysis of the promoter of the human Jagged1 sequence (Katoh and Katoh, 2006). Since elevated Wnt signaling, usually due to mutations in the Apc gene, drive colon tumor development, we evaluated how this was linked to Jagged1 expression. Here we report that in human small intestinal and colonic epithelium, Jagged1 expression was restricted to enteroendocrine cells or was undetectable, respectively, but was elevated in about 50% of human colon tumors. This heterogeneity extended to pronounced differences in elevated expression of Jagged1 between adenomas from the $Apc^{1638N/+}$ and $Apc^{Min/+}$ models of mouse intestinal tumorigenesis, and even among tumors within each congenic strain, therefore indicating potentially stochastic events in linking Wnt signaling to Jagged1

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expression. Further, in HT29Cl16E cells, a human colon carcinoma cell line that retains the ability to differentiate along the goblet cell lineage, we demonstrated that elevated Notch signaling is driven by Jagged1. Since inhibition of Notch signaling has been demonstrated to induce differentiation of intestinal adenoma cells in $Apc^{Min/+}$ mice, our data suggest that this could be accomplished by targeting Jagged1 expression in colon tumors of a subset of patients, providing a therapeutic window in these patients for targeting tumor cells in which Notch signaling shows a much greater dependence on Jagged1 than does Notch signaling in the normal intestinal mucosa.

Results

The *Jagged1* gene, which encodes a Notch ligand, has been reported to be transcriptionally activated by the β-catenin/TCF4 complex, a downstream effector in Wnt signaling (Estrach *et al*., 2006; Katoh and Katoh, 2006). We confirmed that *Jagged1* was also a target of Wnt signaling in colon cells by showing that induction of dominant-negative TCF4 in LS174 T-cells inhibited the expression of Jagged1 (Supplemental Figure 1). Since colon tumorigenesis is frequently driven by elevated Wnt signaling, usually due to mutations in the APC gene, we investigated Jagged1 expression by immunohistochemistry in human tissue. In the normal human small intestinal epithelium, Jagged1 expression was detected in only a limited number of endothelial cells and in vascular smooth muscle (Figure 1a), as previously reported (Sander and Powell, 2004), and in a subset of enteroendocrine cells, as shown by co-staining of these cells for Chromagranin-A and Jagged1 (Figure 1b). However, despite the lack of expression of Jagged1 detected in the normal human colonic mucosa (two examples shown in Figure 1c), we found that 47% of colonic tumors analysed using tissue arrays, were positive (Table 1). Labeling was prominent in large punctuate structures resembling endocytic vesicles, similar to those observed in cells with active Delta–Notch signaling (Ohlstein and Spradling, 2006). In parallel, for these tissues we performed immunostaining for β-catenin, an effector of Wnt signaling that is elevated and often relocalized from the plasma membrane to the nucleus in colon tumor cells. In this set of tissues, 60% of tumors showed loss of plasma membrane staining coincident with elevated cytoplasmic staining for β-catenin (Table 1 and Supplementary Figure 2), with 46% showing distinct nuclear staining (not shown), frequencies similar to those in previous reports (Wheeler *et al*., 2002). Of the 18 tumors positive for elevated β-catenin, nine were positive for Jagged1 staining and nine were negative. Thus, overall, and in relation to detectable changes in cytoplasmic β-catenin, approximately half of the tumors stained positively for expression of Jagged1. In this set of tumors, there was no association of Jagged1 with clinical stage (Table1), although only one of nine metastatic tumors evaluated was positive for Jagged1 expression (not shown), which may reflect the recent suggestion that elevated Notch signaling characterizes the earlier stages of human colon tumor development (Fre *et al*., 2009).

To investigate this heterogeneity of Jagged1 expression further, we turned to two mouse models of intestinal tumors initiated by mutation of the APC gene: the $Apc^{Min/+}$ and $Apc^{1638N/+}$ models, each on a congenic C57Bl/6 background. In each case, it is well documented that loss or inactivation of the wild-type allele elevates Wnt signaling and initiates intestinal adenoma formation (Levy *et al*., 1994; Smits *et al*., 1997). As assayed by replicate quantitative real-time PCR (qRT–PCR) for each tumor for each marker, there was great heterogeneity in Jagged1 expression in the tumors relative to the low levels in the flat mucosa. Figure 2 shows that Jagged1 expression was elevated ($T/N > 1.4$) in 11 of 57 (19%) of $Apc^{Min/+}$ mouse tumors, but in a greater percentage, 9/14 (64%), of tumors from $Apc^{1638N/+}$ mice. Moreover, the mean increase in the $Apc^{1638N/+}$ tumors was 2.6-fold, while in the $Apc^{Min/+}$ mouse tumors there was no increase. Table 2 presents these data in greater detail, and relates the changes in expression of Jagged1 to that of Hes1, a marker of Notch activation. qRT–PCR analysis showed that, while Jagged1 was elevated in 19% of $Apc^{Min/+}$ tumors, it was reduced ($T/N < 0.6$) in 54%. However, Hes1 was elevated in every $Apc^{Min/+}$ tumor in which Jagged1 was elevated, and although Hes1 was also elevated in 13 tumors (23%) in which Jagged1 was not elevated, it was never elevated in the tumors in which Jagged1 was decreased. Of the nine tumors from $Apc^{1638N/+}$ mice in which Jagged1 was elevated, Hes1 was elevated in 7 (78%). In summary, tumors from $Apc^{1638N/+}$ mice exhibit more frequent and higher elevation of Jagged1 than do tumors from $Apc^{Min/+}$ mice, and of the 20 tumors overall in which Jagged1 was elevated, Hes1 was coincidently elevated in 18 (90%).

To investigate a mechanistic link between Jagged1 expression and Notch activation, we turned to HT29Cl16E cells. This is a malignant human colon carcinoma cell line that differentiates, upon contact inhibition of cell growth, into normal-appearing polarized goblet cells that synthesize and secrete colon-specific mucin (Augeron and Laboisse, 1984; Augenlicht *et al*., 1987; Laburthe *et al*., 1989; Merlin *et al*., 1994). Since transition between these states seems to recapitulate the normal growth arrest and lineage-specific differentiation of colonic progenitor cells that is mediated by reduction of Notch signaling in the intestine (Yang *et al*., 2001; Shroyer *et al*., 2007), we focused on this cell line to investigate the potential role of Notch signaling in general, and Jagged1 specifically, in regulating Notch signaling in the colon carcinoma cell phenotype.

During growth arrest and differentiation of the HT29Cl16E cells over a 20-day period, the expression levels of the Notch1 and Notch2 receptors did not change (Figure 3a), although the cleaved activated forms, NICD1 and NICD2, progressively decreased (Figure 3b). Coincident with these decreases, there was a

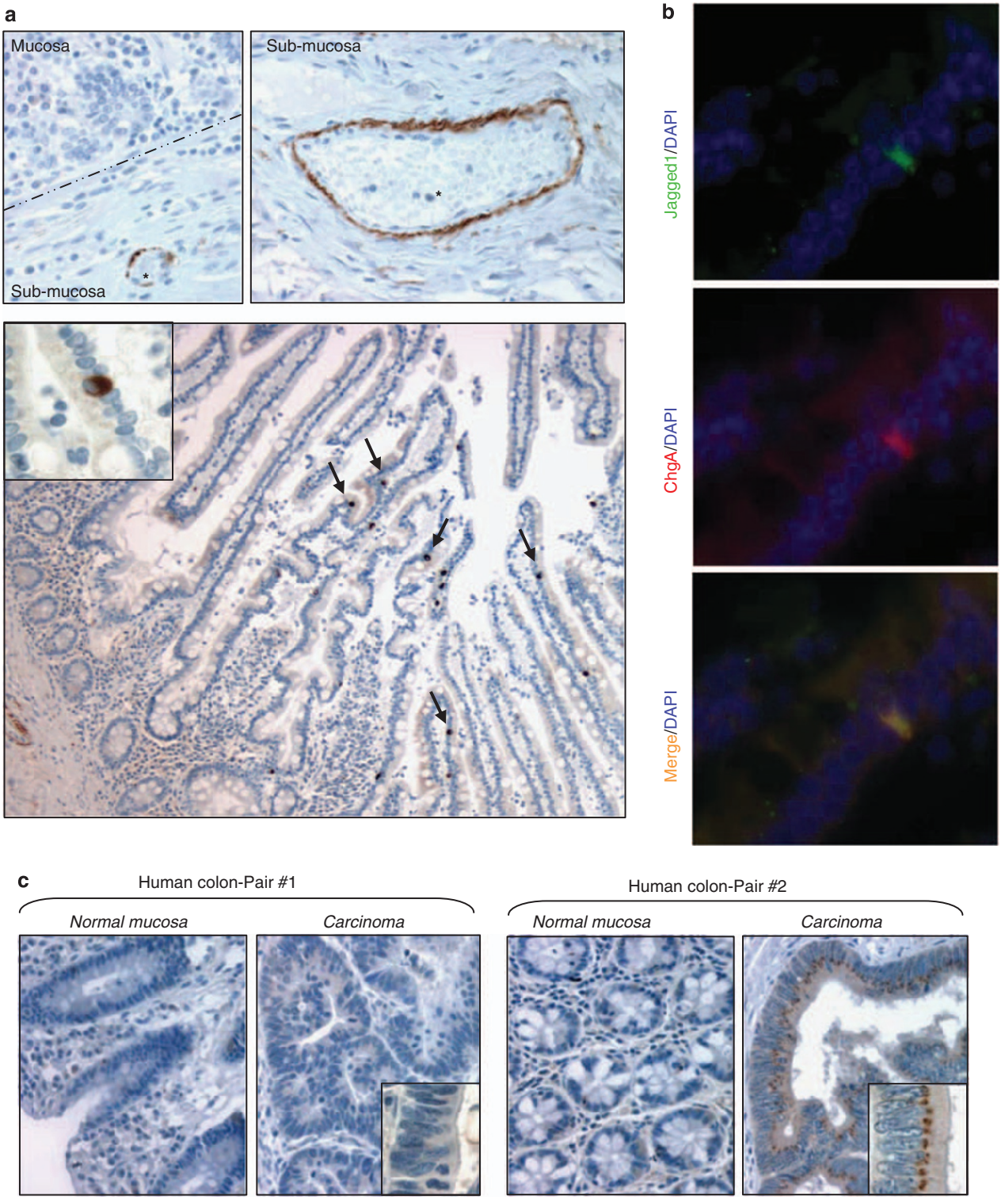

Figure 1 Jagged1 expression is enhanced in human colon cancer. (**a**, **b**) Immunohistochemical staining for Jagged1 was performed on 5-μm human small intestinal serial sections. (**a**) Jagged1 immunostaining in paraffin-embedded human small intestinal sections was detected in endothelial and vascular smooth muscle. Asterisks indicate lumens of blood vessels. (Black arrows indicate cells labeled for Jagged1; inserts are higher magnification images of Jagged1 labeling). (**b**) Triple labeling of intestinal enteroendocrine cells for Jagged1 (fluorescein isothiocyanate, green), Chromogranin-A (ChgA) (Alexa 555, red) and nuclei (4′,6-diamidino-2-phenylindole, blue). (**c**) Jagged1 immunostaining (Novus Biological Inc.) of Imgenex human colon cancer tissue array (IMH-359). Representative images from two pairs of colonic tumor, with the normal adjacent tissue controls.

Table 1 Immunohistochemical staining of Jagged1 and β-catenin expression in human colon carcinomas

Site	*Diagnosis*	*Stage*	*Jagged1 staining*	*β-Catenin staining*
Ascending colon	Normal colon		–	–
Rectum	Normal colon		–	–
Sigmoid colon	Normal colon		–	–
Sigmoid colon	Normal colon		–	–
Cecum	Adenocarcinoma, mod dif	3B	–	+
Ascending colon	Adenocarcinoma, mod dif	3B	–	–
Ascending colon	Adenocarcinoma, mod dif	2A	+	+
Ascending colon	Adenocarcinoma, mod dif	2A	–	+
Ascending colon	Adenocarcinoma, poorly dif	3C	–	–
Ascending colon	Adenocarcinoma, well dif	3B	+	+
Transverse colon	Adenocarcinoma, mod dif	3C	+	+
Transverse colon	Adenocarcinoma, well dif	2A	–	+
Descending colon	Adenocarcinoma, mod dif	3B	+	–
Rectum	Adenocarcinoma, mod dif	2B	–	+
Rectum	Adenocarcinoma, mod dif	3C	–	+
Rectum	Adenocarcinoma, well dif	3B	+	+
Rectum	Adenocarcinoma, well dif	3B	–	+
Sigmoid colon	Adenocarcinoma, mod dif	3C	–	+
Sigmoid colon	Adenocarcinoma, mod dif	3B	+	+
Sigmoid colon	Adenocarcinoma, mod dif	2B	+	–
Sigmoid colon	Adenocarcinoma, mod dif	2A	+	+
Sigmoid colon	Adenocarcinoma, mod dif	2A	+	+
Sigmoid colon	Adenocarcinoma, mod dif	4	+	–
Sigmoid colon	Adenocarcinoma, mod dif	4	+	+
Sigmoid colon	Adenocarcinoma, well dif	3C	–	+
Sigmoid colon	Adenocarcinoma, well dif	2A	+	+
Sigmoid colon	Adenocarcinoma, well dif	3B	–	–
Sigmoid colon	Adenocarcinoma, well dif	3B	–	+
Sigmoid colon	Adenocarcinoma, well dif	4	+	–
Ascending colon	Mucinous carcinoma	2A	–	–

Jagged1 and β-catenin immunostaining of Imgenex human colon cancer tissue array (IMH-359). For histological scoring see section Materials and methods.

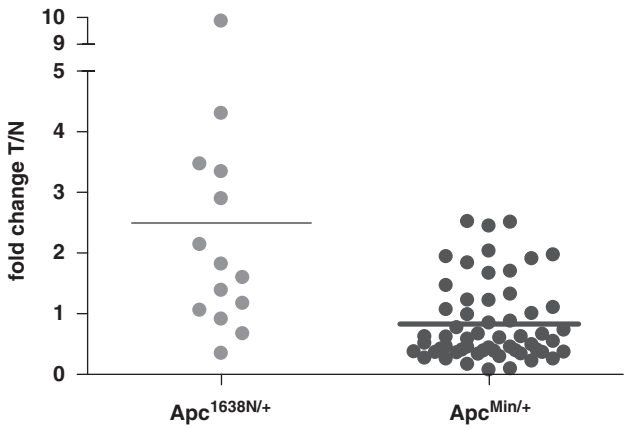

Figure 2 Jagged1 expression is heterogeneous in intestinal tumors from $Apc^{1638N/+}$ and $Apc^{Min/+}$ mice. qRT–PCR analysis of *Jagged1* expression was performed in duplicate in samples extracted from total mucosa of normal small intestine (N) and adjacent tumors (T) from Apc^{1638} and $Apc^{Min/+}$ mice. GAPDH was used as internal control. Data were expressed as fold change T/N. qRT–PCR, quantitative real-time PCR.

marked decrease in the expression of Hes1 (Figure 3b), an immediate target of activated Notch receptors, paralleled by a large increase in the expression of the gene Hath1 (Figure 3c), which is normally repressed by Hes1, and this in turn paralleled the increased expression of the goblet cell markers trefoil factor-3 (Tff3) and Muc2 (Figure 3b). Thus, maturation of these cells *in vitro* into mature goblet cells was indeed accompanied by decreased canonical Notch signaling.

To confirm that altered signaling through Notch was in fact necessary for maturation of these cells, we treated the HT29Cl16E cells with L-685,458, an inhibitor of γ-secretase activity, which interferes with proteolysis and subsequent activation of all Notch receptors (Martys-Zage *et al*., 2000). Inhibition of Notch signaling by this γ-secretase inhibitor (GSI) was first assessed in cells transfected with an RBP-Jκ-dependent regulatory element, the TP1 promoter–luciferase reporter construct. After 10-μM GSI treatment of subconfluent HT29Cl16E cells, TP1 luciferase-reporter activity was decreased by 52% as compared with that in dimethylsulfoxide-treated cells at 48 h (Figure 4a), Hes1 expression was repressed (Figures 4a and b) and *Hath1* mRNA level increased more than three-fold as compared with that in dimethylsulfoxide-treated cells at 24 h (Figure 4b). This was accompanied by a 46% decrease in cell number (Figure 4c), suggesting that inhibition of Notch signaling decreased cell proliferation and/or cell survival. Using fluorescence-activated cell sorting to analyse the cell cycle, we confirmed that GSI inhibited the percentage of cells in S-phase by nearly 50% and increased the number of cells in the G_0/G_1 fraction from 41 to 63% (Figures 4d and e), consistent

Table 2 Jagged1 and Hes1 fold change in tumors versus normal tissue from mouse small intestine

Apc$^{Min/+}$			*Apc*$^{1638N/+}$		
Tumor	*Jagged1 T/N*	*Hes1 T/N*	*Tumor*	*Jagged1 T/N*	*Hes1 T/N*
1	0.66	1.16	1	1.06	2.18
2	1.1	1.28	2	2.14	1.73
3	1.97	1.83	3	1.81	1.86
4	1.22	1.76	4	4.3	2.6
5	1.22	1.67	5	3.47	3.36
6	0.49	1.03	6	0.91	3.67
7	0.6	1.08	7	9.86	2.18
8	0.84	1.47	8	2.89	1.17
9	0.4	0.92	9	1.59	1.25
10	0.39	0.83	10	3.34	4.1
11	0.62	0.87	11	1.38	2.13
12	0.73	1.43	12	0.67	1.36
13	0.4	1.33	13	1.17	3.22
14	0.22	0.77	14	0.35	0.5
15	0.45	0.79			
16	0.36	0.52			
17	0.36	0.89			
18	1.02	1.05			
19	2.03	3.51			
20	2.52	3.55			
21	2.44	1.93			
22	1.7	2.88			
23	1.9	2.34			
24	1.94	3.48			
25	2.51	3.09			
26	1.32	3.53			
27	1.46	2.66			
28	1.66	1.9			
29	0.98	1.95			
30	0.54	8.18			
31	0.46	3.69			
32	0.36	3.61			
33	0.43	5			
34	0.51	4.98			
35	0.87	3.69			
36	0.58	4.13			
37	1.83	7.22			
38	0.62	4.97			
39	0.33	0.45			
40	0.37	1.29			
41	0.61	0.75			
42	0.37	0.72			
43	0.38	1.2			
44	0.41	0.96			
45	0.39	0.8			
46	0.34	0.43			
47	0.27	0.47			
48	0.45	0.4			
49	0.66	1.33			
50	0.29	0.6			
51	0.25	0.73			
52	0.77	1.27			
53	0.16	0.53			
54	1.06	0.39			
55	0.25	0.52			
56	0.07	0.3			
57	0.09	0.27			

Abbreviation: Hes, Hairy Enhancer of Split.
Quantitative real-time PCR analysis of *Jagged1* and *Hes1* expression was performed in duplicate in samples extracted from total mucosa of normal small intestine (N) and adjacent tumors (T) from Apc$^{1638N/+}$ and Apc$^{Min/+}$ mice. GAPDH was used as internal control. Data were expressed as fold change T/N.

with a G_1 cell-cycle arrest upon inhibition of Notch signaling. In parallel, GSI treatment resulted in 329 and 278% increases of *Tff3* and *Muc2* mRNA levels, respectively, at 48 h, reflecting induction of the transcriptional differentiation programme of these cells (Figure 4b). Thus, canonical Notch signaling was necessary for maturation of these cells along the goblet cell lineage.

To target the effects of Notch more specifically, we reduced endogenous Hath1 expression by 34% using a small interfering RNA (siRNA) approach. This was sufficient to significantly repress *Tff3* and *Muc2* mRNA expression in differentiating HT29Cl16E cells (Supplementary Figure 3). Since levels of the activated forms of Notch1 and Notch2 (NICD1 and NICD2, respectively) both decreased during 16E cell differentiation, we analysed the contribution of these receptors to regulation of proliferation and differentiation of these colon cancer cells. siRNA targeted to Notch1 reduced Notch1 mRNA (42% inhibition at 48 h as compared with cells treated with non-targeting siRNAs) and protein expression (Figure 5a). This reduced Hes1 transcript levels by only approximately 25%, but this did not significantly alter the expression of *Hath1* or the expression of the differentiation markers *Tff3* and *Muc2* (Figure 5a), nor did this alter proliferation (not shown). There was a similar lack of effect on HT29Cl16E cell maturation following a 65% decrease of endogenous Notch2 expression using Notch2-specific siRNA (Figure 5b). We also used lentiviral transduction of two different short-hairpin RNAs (shRNAs) targeted to Notch1 (vectors A and B) or to Notch2 (vectors C and D), and found reduction of clonogenic growth of 25% with the former and 10–20% with the latter, demonstrating that inhibition of Notch1 or Notch2 individually had only modest effects on clonogenicity (Supplemental Figure 4). However, when the Notch1 and Notch2 receptors were targeted by their cognate siRNAs simultaneously, there was, 48 h after transfection, a 32 and 51% reduction of *Notch1* and *Notch2* mRNA levels, respectively, which significantly enhanced *Hath1*, *Tff3* and *Muc2* expression by 72 h (Figure 5c). Thus, the two receptors appeared to act redundantly in the transformed HT29Cl16E cells, similar to their role in the normal intestinal epithelium (Riccio *et al*., 2008). Interestingly, although the goblet cell differentiation programme was effectively triggered by simultaneous downregulation of Notch1 and Notch2 by siRNA, there was no effect on cell proliferation (not shown), in contrast to the shRNA experiments that showed each to have modest effects on clonogenic growth. Thus, the effects of Notch signaling on growth and differentiation may be distinct, either due to threshold levels for effect or pathways involved (see Discussion).

Reduction in Notch signaling was, therefore, necessary for differentiation of these cells, but the important question remained as to what drives Notch signaling in the undifferentiated, proliferating, malignant HT29Cl16E cells? Since we have previously shown that Wnt signaling, a putative inducer of Jagged1, decreased

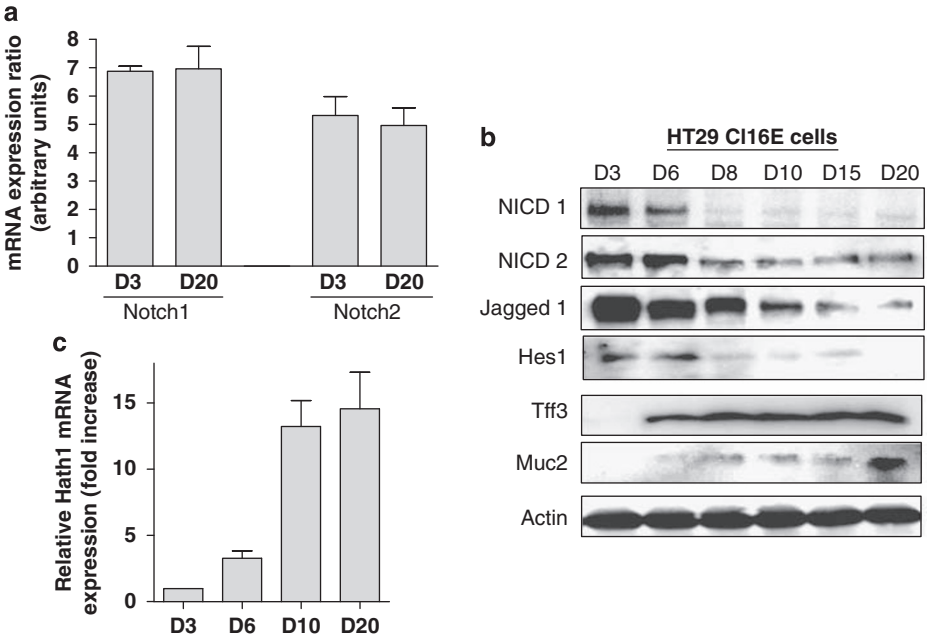

Figure 3 The canonical Notch signaling pathway is downregulated during spontaneous differentiation of HT29 Cl16E cells. qRT-PCR analysis of *Notch1*, *Notch2* (**a**) and *Hath1* (**c**) expression was performed using 16E cells at the indicated times after seeding. Data were expressed as mean fold increase ± s.e.m. (**b**) Total protein extracts from 16E cell lysates, harvested at 3–20 days after seeding, were fractionated by sodium dodecyl sulfate–PAGE and analysed by western blot for expression of NICD1 (110 kDa), NICD2 (110 kDa), Jagged1 (140 kDa) and the Notch canonical target Hes1 (30 kDa). Tff3 (7 kDa) and Muc2 (> 500 kDa) expression were used as a validation for spontaneous differentiation of 16E toward the goblet cell lineage and β-actin was used as loading control. Hes, Hairy Enhancer of Split; NICD, Notch intracellular domain; qRT–PCR, quantitative real-time PCR; Tff, trefoil factor.

during spontaneous growth arrest and differentiation of these HT29Cl16E cells (Velcich *et al*., 2005), we investigated the role of Jagged1. Figure 3b shows that Jagged1 levels decreased along with downregulation of NICD1 and NICD2, and Hes1, which paralleled our previously reported decreases in Wnt signaling and tumorigenicity of the HT29Cl16E cells (Velcich *et al*., 2005), consistent with a role for Jagged1 in active Notch signaling in the transformed cells. To investigate this, endogenous *Jagged1* mRNA expression was reduced by 45%, 72 h after transfection of siRNA targeting *Jagged1* (Figure 5d). This was sufficient to significantly decrease Jagged1 protein (Figure 5d, insert) and *Hes1* mRNA expression (Figure 5d), demonstrating that Notch-signaling pathway activation in the proliferating malignant cells was reduced by cell-autonomous downregulation of Jagged1. This downmodulation of *Jagged1* in 16E cells resulted in increased *Hath1*, *Tff3* and *Muc2* mRNA expression (~3 fold), as compared with that in cells transfected with a non-targeting siRNA (Figure 5d). Again, however, this downregulation of Notch signaling by targeting of the ligand did not affect cell proliferation, assayed by either 5-bromo-2-deoxyuridine incorporation or total number of cells (data not shown), although lentivirus-mediated shRNA to Jagged1 targeted by two different vectors reduced clonogenicity by 40–50% ($P<0.001$; Supplementary Figure 4), emphasizing the potential dichotomy of the effects of Notch signaling on lineage-specific differentiation and proliferation (Discussion). Thus, not only does canonical Notch signaling persist in this human colon carcinoma cell line, but it is driven by Jagged1 expression that can be targeted to force the cells to differentiate through a Notch-signaling-dependent mechanism.

Discussion

Notch signaling is an attractive target for colon tumor therapy and prevention since the pathway has been shown recently to be elevated frequently in mouse and human intestinal tumors (Fre *et al*., 2008, 2009), and its inhibition can force the differentiation of tumor cells in $Apc^{Min/+}$ intestinal adenomas *in vivo* at low efficiency (van Es *et al*., 2005), although it has been reported recently that this can significantly reduce tumor size and incidence in $Apc^{Min/+}$ mice (Ghaleb *et al*., 2008; Rodilla *et al*., 2009). However, a limiting factor in the clinical targeting of the Notch pathway is major gastrointestinal toxicity, as Notch downregulation is also fundamental in driving secretory cell differentiation in the normal intestinal mucosa (Jensen *et al*., 2000; van Es *et al*., 2005; Guilmeau *et al*., 2008; Riccio *et al*., 2008). Thus, short-term inactivation of the Notch pathway, either genetically or pharmacologically, causes a secretory cell metaplasia, and persistence of the goblet cell metaplasia over 9 months in the mouse results in a major inflammatory response, which we believe involves

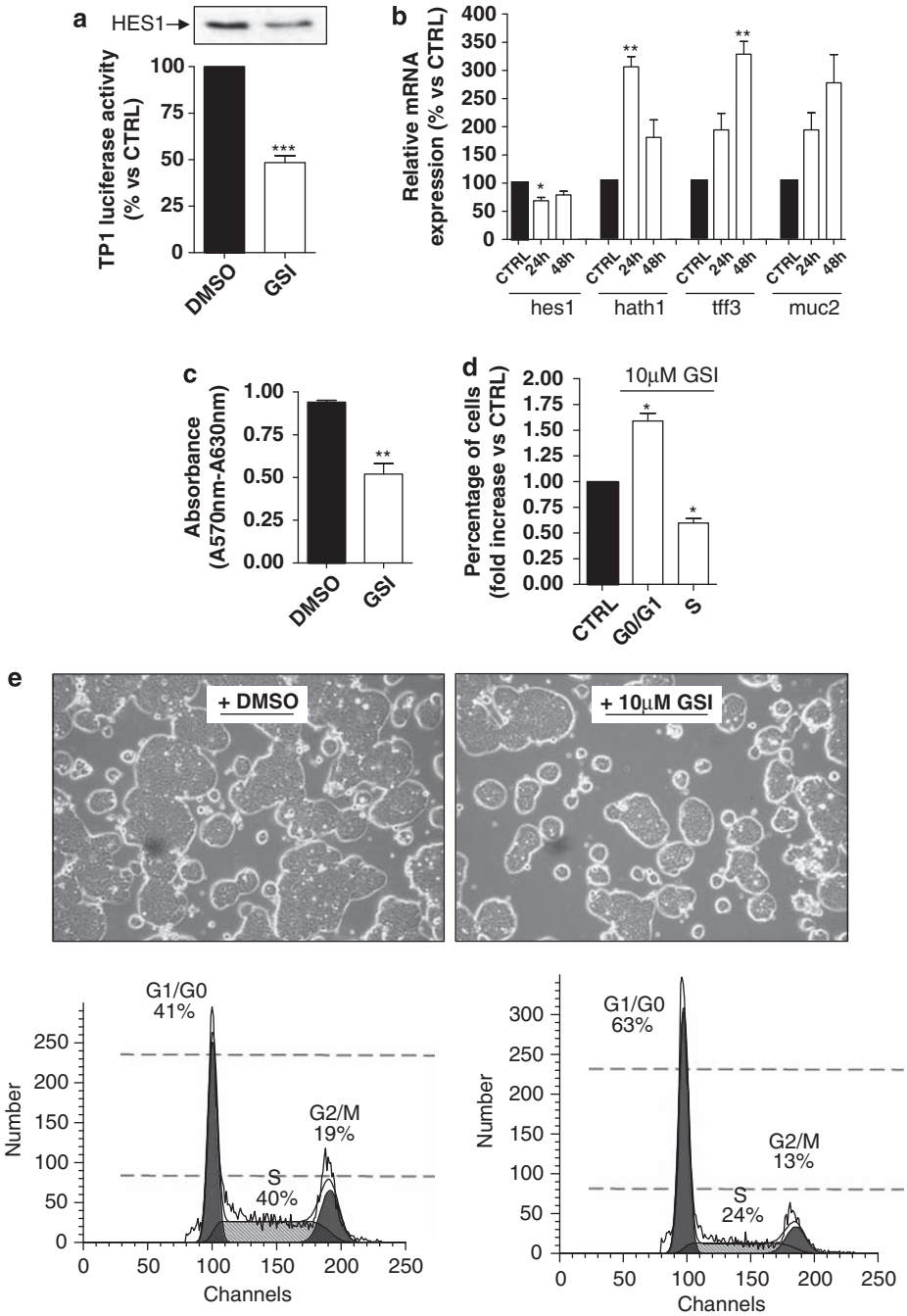

Figure 4 Inhibition of γ-secretase activity induces growth arrest and expression of goblet cell marker in HT29 Cl16E colon cancer cells. Subconfluent 16E cells were cultured with 10 μM of GSI or the equivalent volume of dimethylsulfoxide and were harvested 24, 48 or 72 h later. (**a**) A transient reporter assay with a TP1-Luc reporter plasmid was performed and after 48 h of treatment; fold induction of luciferase activity for each sample was calculated compared with the control. (**a**, insert) Aliquots of the cells described above were lysed and total cell extracts were immunoblotted with Hes1 antibody (generous gift from T Sudo). (**b**) qRT–PCR analyses using specific human primers were performed to determine the relative mRNA abundance of *Hes1*, *Hath1*, *Tff3* and *Muc2*. Expression of each transcript was normalized to *GAPDH* and expressed as a percentage compared to dimethylsulfoxide-treated cells ($n = 3$). Data are expressed as mean ± s.e.m. (**c**) Cell proliferation rate was determined by MTT assay after 48 h of treatment. (**d**) DNA content was analysed by flow cytometry (right panel). Each bar represents the mean ± s.e. of three experiments. (**e**) Representative fluorescence-activated cell sorting profile of 16E cells stained with propidium iodide after 48 h of GSI treatment, and the corresponding percentage of cells with G_0/G_1, S and G_2/M-phase DNA content. GSI, γ-secretase inhibitor; Hes, Hairy Enhancer of Split; qRT–PCR, quantitative real-time PCR; Tff, trefoil factor.

sequestration of commensal bacteria in the proximity of the mucosal surface due to binding or trapping by the extensive mucous secretion (Guilmeau *et al*., 2008).

In this study, we report that the expression of the Notch ligand, Jagged1, was restricted to enteroendocrine cells in normal human intestinal epithelium,

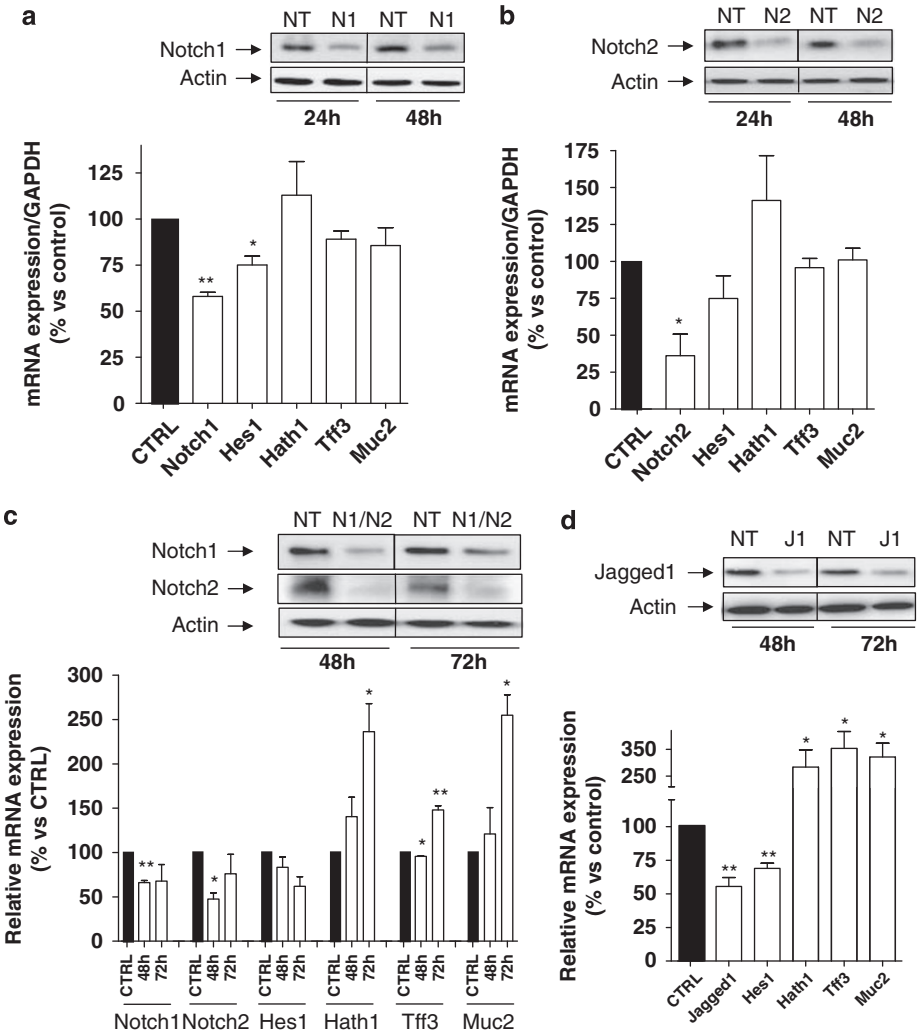

Figure 5 Jagged1 inhibition induces precocious expression of Tff3 and Muc2 in HT29 Cl16E cells. (**a**, **b**) Subconfluent 16E cells were transfected with siRNA to *Notch1* (50 nM) or to *Notch2* (100 nM) (designated as N1 or N2, respectively) or the corresponding concentration of non-targeting siRNA (termed NT), as control. Lysates were analysed 48 h post transfection by qRT–PCR for the relative abundance of *Hes1*, *Hath1*, *Tff3* and *Muc2* mRNA ($n = 3$). (**c**) siRNAs targeting *Notch1* or *Notch2* (each 50 nM) were transfected simultaneously into subconfluent 16E cells. A 100-nM concentration of non-targeting siRNA was used as control. (**d**) Bars indicate relative expression of *Hes1, Hath1, Tff3* and *Muc2*, as determined by quantitative real-time PCR, using 16E cells 72 h after transfection of 100 nM anti-Jagged1 siRNA ($n = 4$). (**a**–**d**) Expression of each transcript was normalized to GAPDH and calculated as a percentage of control. Data are expressed as mean ± s.e.m. (Insets) Western blot analysis of Notch1 (~300 kDa), Notch2 (~300 kDa) and Jagged1 (140 kDa) expression levels were performed in parallel. Hes, Hairy Enhancer of Split; qRT–PCR, quantitative real-time PCR; Tff; siRNA, small interfering RNA; Tff, trefoil factor.

but that its expression was elevated in ~50% human colon tumors, and in mouse $Apc^{1638N/+}$ intestinal tumors, but less frequently in tumors of $Apc^{Min/+}$ mice. Therefore, targeting of Jagged1 in a subset of patients may provide a therapeutic window for inhibiting Notch signaling in colon tumors, while sparing effects on the normal mucosa. The restricted expression of Jagged1 in the normal intestinal mucosa may result from random asymmetric division of intestinal secretory precursors, as described for another Notch ligand, Delta, in adult *Drosophila* midgut (Ohlstein and Spradling, 2006).

There was not a direct correspondence between β-catenin nuclear translocation in the tumors and Jagged1 expression, despite the fact that data in the literature suggest, and *in vitro* data reported here demonstrate, that Jagged1 can be regulated by Wnt signaling in colon epithelial cells (Estrach *et al*., 2006; Katoh and Katoh, 2006), conclusions that were extended by Rodilla *et al*. (2009) while this paper was under review. Moreover, we observed that this heterogeneity in Jagged1 expression extended to tumors in the congenic $Apc^{Min/+}$ and $Apc^{1638N/+}$ models, two mouse strains in which elevated Wnt signaling initiates intestinal tumor development. Thus, not only may there be specific mechanisms that target the components of Notch signaling, such as Jagged1, during tumorigenesis, but there may be stochastic events that determine the level of expression as well as the role played by individual components in specific tumors.

Importantly, we found that in HT29Cl16E colonic carcinoma cells, differentiation into goblet cells and clonogenic growth were dependent on downregulation of Jagged1 expression that was driving Notch signaling. However, it was interesting that inhibition of Notch signaling with GSI inhibited the proliferation and induced the expression of secretory markers, whereas downregulating Notch by simultaneously targeting Notch1 and Notch2 with an siRNA approach, or Jagged1, was sufficient to induce the markers of goblet cell differentiation, but failed to alter the proliferation of the HT29Cl16E cells. Similar discrepancies were observed between GSI treatment and Notch/RBP/Jκ pathway silencing in taxane-induced mitotic arrest in colon cancer cells (Akiyoshi *et al*., 2008), suggesting that other targets of γ-secretase activity (McCarthy, 2005) might be involved in cell-cycle regulation of HT29Cl16E colon cancer cells. This may be related to the recent report that cells respond to a Notch-dependent mitogenic stimulus only when the Wnt cascade is intact, but that the effect of Notch on cell differentiation is independent of Wnt (Fre *et al*., 2009). Thus, in the growing HT29Cl16E colon carcinoma cells, lack of effect on proliferation by siRNA reduction of Notch1, Notch2 or Jagged1, but modulation of differentiation, could be due to altered Wnt signaling. Similar results were reported by Rodilla *et al*. (2009) who showed that expression of the active signal NICD1 in Ls174-dnTCF4 cells is able to regulate differentiation but not proliferation. Alternatively, lack of growth inhibition following silencing of Notch1 and Notch2, or of Jagged1, could be due to dosage effects: more efficient downregulation of these drivers of Notch signaling by either GSI or shRNA appears also to inhibit proliferation, and thus this may involve different thresholds for the effects of Notch signaling on differentiation versus growth. Regardless of the underlying explanation, the data demonstrate that the effects of Notch on differentiation and proliferation can be dissociated, emphasizing that Notch provides only one set of signals that must be integrated in establishing mucosal homeostasis.

In summary, despite the fundamental role that Notch signaling plays in maintaining a proliferating progenitor cell phenotype in the normal intestinal mucosa, and its role in tumorigenesis, it is likely that, as with most biomarkers of transformation, effective therapeutic targeting will require multiple strategies based on both the role of the pathway and the mechanisms underlying its deregulation, in different subsets of colorectal tumors.

Materials and methods

Animals and Tissue preparation

$Apc^{Min/+}$ (Moser *et al*., 1990) and $Apc^{1638N/+}$ (Fodde *et al*., 1994) mice on C57BL/6 background were treated according to animal protocols approved by the Animal Care and Use Committee at Montefiore Medical Center and the Albert Einstein College of Medicine. Small and large intestine were removed immediately after killing the mice at 5 or 8 months of age ($Apc^{Min/+}$ and $Apc^{1638N/+}$, respectively), flushed gently with cold phosphate-buffered saline, opened longitudinally and inspected with a dissection microscope. Tumors, and adjacent flat mucosa, were dissected, homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA) and processed for mRNA extraction.

Cell culture

Cell lines were maintained at 37 °C under 5% CO_2. HT29 Cl16E cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 5% glutamine, and LS174T-dnTCF4 cells in RPMI1640 (Gibco BRL, Grand Island, NY, USA). Media contained 10% fetal bovine serum and 1% penicillin/streptomycin (100 U of penicillin and 100 μg of streptomycin per ml) and 10 mM HEPES buffer. For *in vitro* spontaneous differentiation, HT29 Cl.16E cells were seeded at a density of 10^6 cells per 25-cm^2 flask and used 3, 6, 8, 10, 15 or 20 days after plating.

Immunohistochemistry and histological scoring

Intestinal and colonic tissues were routinely fixed overnight in 10% neutral buffered formalin and embedded in paraffin. After de-waxing and hydration, 5-μm sections were pretreated with 3% H_2O_2 for 10 min at room temperature and incubations with rabbit anti-Jagged1 primary antibody (Novus Biological Inc., Littleton, CO, USA, dilution 1:1000) or with mouse anti-β-catenin primary antibody (BD Transduction Laboratories, San Jose, CA, USA, C192, dilution 1:5000) were performed overnight at 4 °C. Antigen was retrieved by boiling sections in 10 mM citrate buffer (pH 6.0, 30 min). Immunodetection was performed with biotinylated anti-rabbit or anti-mouse antibodies using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and 3,3-diaminobenzidine hydrochloride (Sigma, St Louis, MO, USA) as a peroxidase chromogen. The specificity of the reaction was tested by omitting the primary antibody. Nuclei were counterstained with Mayer's hematoxylin.

Evaluation of Jagged1 staining in human tumors (Table 1) was either completely negative or showed many positive cells. Therefore, it was possible to use a simple dichotomous score of minus (−) or plus (+) to characterize Jagged1 expression in these human tumors. For β-catenin, tumors scored negative (−) exhibited plasma membrane staining, with low levels of cytoplasmic staining, while those scored positive (+) exhibited loss of plasma membrane staining coincident with elevated cytoplasmic staining.

Cell proliferation assay and fluorescence-activated cell sorting analysis

The colorimetric MTT assay, using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, was performed according to the manufacturer's recommendations (Sigma). For flow cytometric analysis of cell-cycle distribution, both adherent cells and cells in the culture media were harvested by centrifugation. After staining with propidium iodide, the G_0/G_1, S, G_2/M and the sub-G_1 fractions were analysed with a FACSCalibur flow cytometer (Beckton Dickinson, San Jose, CA, USA). Cell-cycle distribution was determined using CELL Quest, version 3.3, and Modfit software.

Cell culture with siRNAs and lentiviral transduction of shRNAs

For siRNAs, the cells were transfected using Dharmacon siGENOME SMARTpool reagents for human *Notch1*, *Notch2* and *Jagged1*. Twenty-four hours after seeding, subconfluent cells were transfected with gene-specific siRNAs using

Lipofectamine 2000 as a carrier, according to the manufacturer's instructions (Invitrogen Inc.). Two control transfections were performed, one with Lipofectamine 2000 alone and the other with Dharmacon siCONTROL non-targeting siRNA (catalog number D-001206-13-05). The cells were exposed to the transfection mixture for 6 h, then cultured in regular growth medium for 24, 48 or 72 h, and finally harvested and stored at −80 °C.

For shRNAs, the cells were infected using shRNA lentiviral particles according to the manufacturer's instructions (Sigma-Aldrich, St Louis, MO, USA). The shRNA lentiviral particles used were the following: SHC002 V (non-targeting control, A and B) SHCLNV-NM_017617 clones TRCN0000003360 and TRCN0000003359; (Notch1; C and D) SHCLNV-NM_024408 clones TRCN0000004896 and TRCN0000004895; and (Notch2; E and F) SHCLNV-NM_000214 clones TRCN00000 33441 and TRCN0000033443. Selection was performed on growing infected cells for 7 days in medium supplemented with 5 μg/ml of puromycin.

Clonogenic assay
HT29 Cl16E cells previously infected with the specific or control shRNAs lentiviral particles and selected with puromycin, were seeded at a density of 800 cells per well of a six-well plate and grown for 21 days. Colonies were fixed and stained with 6% glutaraldehyde and 0.5% crystal violet, and counted using the Total Lab 1.1 software (Nonlinear Dynamics, Durham, NC, USA).

Plasmid constructs, cell transfection and reporter assays
The TP1-Luc Notch reporter construct was previously described by Takebayashi *et al*. (1994) and Strobl *et al*. (1997). To perform luciferase-reporter assays, subconfluent cells were cotransfected 24 h after seeding, using Lipofectamine 2000 reagent (Invitrogen) with 0.2 μg of reporter plasmid and 0.05 μg of the pRL-TKRenilla luciferase control plasmid. After 16 h at 37 °C, the transfection medium was replaced by culture medium containing 10 μM of L-685,458 or an equal volume of the dimethylsulfoxide solvent. Firefly and *Renilla* luciferase activities were determined in whole-cell extracts 48 and 72 h after transfection using the Dual Luciferase reporter assay (Promega, Madison, WI, USA) and a Turner Designs TD200 dual luminometer. All experiments were performed three times in triplicate.

Protein extraction, western blot and antibodies
For whole-cell protein extraction, cell pellets were homogenized at 4 °C in lysis buffer supplemented with a cocktail of protease inhibitors (P8340; Sigma). Homogenates were centrifuged at 15 000 *g* for 15 min at 4 °C, and supernatants collected for western blot analysis. Protein concentration was quantified by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Solubilized proteins were resolved by electrophoresis on sodium dodecyl sulfate–PAGE gels, transferred onto activated polyvinylidene difluoride membranes and subjected to immunoblot analysis. The immune complexes were detected by ECL. Equal protein loading was verified by re-probing membranes with anti-β-actin.

Commercially available antibodies were the following: rabbit anti-Jagged1 (Novus Biologicals Inc.), rabbit anti-Notch1 (Abcam Inc., Cambridge, MA, USA), rabbit anti-Notch2 (Rockland Inc., Gilbertsville, PA, USA), rabbit anti-NICD1 (Cell Signaling Technology, Inc. Denvers, MA, USA), rabbit anti-NICD2 (Rockland Inc.), mouse anti-Tff3 clone 15C6/IgG1 (585350; Calbiochem, San Diego, CA, USA), mouse anti-Muc2 (ab 11197; Abcam Inc.), goat anti-β-actin (sc-1615; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) and mouse anti-β-catenin (Transduction Laboratories, C192).

RNA isolation and quantitation of steady-state mRNA by real-time PCR
Total RNA was extracted from cell pellets or frozen tissues with Trizol reagent (Invitrogen). Briefly, first-strand cDNA was synthesized from 1 to 5 μg total RNA with 200 U of reverse transcriptase using the Superscript II kit (Invitrogen) according to the manufacturer's recommendations. cDNA was amplified using the SYBR Green PCR Master Mix and the ABI PRISM 7900HT Sequence Detection System real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primer sequences (available upon request) were designed using the Primer Express software (Applied Biosystems). A standard curve was generated by real-time PCR analysis of fivefold serial dilutions of a standard template DNA. All reactions were performed in duplicate. The relative values for each PCR product were expressed in arbitrary units as a ratio of the target transcript normalized to human *GAPDH*.

Statistical analysis
Results in the figures are expressed as mean ± s.e.m. Values were compared by Student's *t*-test (two-tailed, assuming equal variances; $*P<0.05$, $**P<0.005$, $***P<0.001$) to test for significant differences.

Conflict of interest

The authors declare no conflict of interest.

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