Modulation of Notch Signaling by Antibodies Specific for the Extracellular Negative Regulatory Region of NOTCH3*


From Tanox, Incorporated, Houston, Texas 77025 and the Department of Pathology, Brigham and Women’s Hospital, Boston, Massachusetts 02115

The Notch pathway regulates the development of many tissues and cell types and is involved in a variety of human diseases, making it an attractive potential therapeutic target. This promise has been limited by the absence of potent inhibitors or agonists that are specific for individual human Notch receptors (NOTCH1–4). Using an unbiased functional screening, we identified monoclonal antibodies that specifically inhibit or induce activating proteolytic cleavages in NOTCH3. Remarkably, the most potent inhibitory and activating antibodies bind to overlapping epitopes within a juxtamembrane negative regulatory region that protects NOTCH3 from proteolysis and activation in its resting auto-inhibited state. The inhibitory antibodies revert phenotypes conveyed on 293T cells by NOTCH3 signaling, such as increased cellular proliferation, survival, and motility, whereas the activating antibody mimics some of the effects of ligand-induced Notch activation. These findings provide insights into the mechanisms of Notch autoinhibition and activation and pave the way for the further development of specific antibody-based modulators of the Notch receptors, which are likely to be of utility in a wide range of experimental and therapeutic settings.

The mature Notch heterodimer is held in an auto-inhibited state by a juxtamembrane negative regulatory region (NRR) consisting of three Lin12/Notch repeats (LNR1–3) and a heterodimerization (HD) domain (4), which is divided into N- (HD1) and C-terminal (HD2) halves by cleavage at site S1 (3, 4). Through an uncertain mechanism, binding of ligands of the Delta/Serrate/Lag-2 (DSL) family to the N-terminal, EGF-repeat region of NOTCH3 elicits this inhibition and induces two successive additional cleavages within the N1/2 subunit that are catalyzed by ADAM-type metalloproteases (5, 6) and γ-secretase (7), respectively. The latter cleavage releases the intracellular domain of Notch (ICN), permitting it to translocate to the nucleus (8, 9), where it forms a transcriptional activation complex with the DNA-binding factor CBF-1/Su(H)/Lag-1 (CSL) and co-activators of the Mastermind family (10, 11).

NOTCH1–4 have broad, overlapping patterns of expression in embryonic and adult tissues and fulfill non-redundant roles during hematopoietic stem cell specification (12) and T-cell (13), marginal zone B-cell (14), renal (15), and vascular development (16). In line with these distinct functions, inherited defects in Notch receptors or ligands cause a number of human developmental disorders, including congenital bicuspid aortic valve (NOTCH1) (17), CADASIL (congenital autosomal dominant strokes, infarcts, and leukoencephalopathy; NOTCH3) syndrome (18), and Alagille syndrome (JAGGED1 and NOTCH2) (19–21). Acquired abnormalities involving specific Notch receptors have also been implicated in human cancers. The best example is T-cell acute lymphoblastic leukemia, which is commonly associated with acquired gain-of-function mutations in NOTCH1 (22), but other Notch receptors are also suspected to act as oncoproteins. For example, activated NOTCH3 is a potent inducer of leukemia in murine models (23, 24) and is overexpressed or amplified in various human solid tumors, including non-small cell lung cancer and ovarian cancer (25, 26). Of note, NOTCH3-deficient mice are viable and fertile (27), suggesting that NOTCH3-selective inhibitors should be well tolerated.
NOTCH3 Antibodies and Their Implications for Notch Regulation

The fortuitous development of γ-secretase inhibitors as possible therapeutic agents in Alzheimer disease has enabled clinical trials of these drugs in cancers associated with hyperactive Notch signaling, such as T-cell acute lymphoblastic leukemia. However, such compounds inhibit the proteolysis of multiple transmembrane proteins, including all four Notch receptors (28), and cause significant toxicities when given chronically, most notably severe secretory diarrhea that results from colonic goblet cell metaplasia (29). This gastrointestinal toxicity is believed to stem from inhibition of NOTCH1 and/or NOTCH2 (30), which promote the differentiation of progenitor cells in the colonic crypts into absorptive enterocytes.

Soluble Notch agonists are also of potential interest for purposes of tissue engineering (e.g. to grow T cells (31) and stem cells) and other applications related to cell growth, development, and immunity function. However, soluble ligands generally appear to behave as antagonists rather than agonists of Notch signaling unless they are fixed to plates or multimerized (32–36), and apparently lack specificity for individual Notch receptors.

In principle, specific inhibitors of individual Notch receptors may avoid or reduce the therapeutic complications caused by non-selective Notch inhibitors, and selective soluble Notch agonists would be useful experimental tools with possible therapeutic applications. In the studies reported here, we describe the discovery of monoclonal antibodies (mAbs) that appear to specifically inhibit or activate NOTCH3. Beginning with unbiased screens, we identified two potent blocking antibodies and one apparent activating antibody, each of which binds to overlapping epitopes on one face of the NRR of NOTCH3. These antibodies are highly specific for NOTCH3 and appear to mimic or inhibit certain effects of DSL ligands on cells. Our findings further highlight the importance of the NRR in regulating Notch activation and establish an avenue for the development of new, specific therapeutic agents directed at this important pathway.

EXPERIMENTAL PROCEDURES

Cell Lines and cDNAs—A full-length NOTCH3 cDNA was generated by RT-PCR using human thymus RNA (Ambion, Inc., Austin, TX) as a template. JAGGED1 and NOTCH3 cDNAs were obtained from a commercial source (OriGene, Rockville, MD). Human JAGGED2 cDNA was generated by RT-PCR using RNA isolated from the human T-cell leukemia cell line HH (ATCC# CRL-2105). cDNAs for NOTCH3, JAGGED1, JAGGED2, ΔEGF-N3 (a construct encoding a fragment of NOTCH3 lacking the ligand binding EGF repeats), and LD-TM (a construct encoding the NOTCH3 leader peptide fused to the residues spanning the NRR region and the transmembrane domain (residues 1378–1711)) were cloned into pcDNA3.1-Hyg (Invitrogen). Stable cell lines carrying pcDNA3.1-Hyg plasmids were generated from 293T cells (ATCC, Manassas, VA). “Tet-on” U2OS flp-in cells (the kind gift of Dr. Jeff Parvin) were co-transfected with pOG44 (which encodes Flp recombinase) and pcDNA5-FRT-TO plasmids encoding the ectodomains and transmembrane domains of human NOTCH1–3 fused to the DNA-binding domain of Gal4 and the transcriptional activation domain of NOTCH1. OP9 control cells and OP9-DLL1 expressing feeder cells were the kind gift of J. C. Zuniga-Pflucker (31).

Generation of NOTCH3 Ectodomain mAbs—cDNAs encoding (i) the NRR region (residues 1378–1640) and (ii) the EGF repeat region (residues 43–1377) of NOTCH3 were ligated in-frame between the NOTCH3 signal peptide sequence and the 5′-end of a cDNA encoding the Fc of human IgG1. The resulting chimeric cDNAs were cloned into pcDNA3.1 and expressed in 293T cells. The secreted fusion proteins were purified from conditioned media by protein A affinity chromatography (Pierce) and injected into male A/J mice (Harlan, Houston, TX), which were subsequently harvested for hybridoma generation (37).

Enzyme-linked Immunoabsorbent Assays—ELISA was performed as described (37). mAbs were mixed with 50 μl of blocking buffer and added to a well coated with immunogen, which was washed and incubated with horseradish peroxidase (HRP)-conjugated, Fc-specific goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min. The HRP substrate solution containing 0.1% 3,3,5,5-tetramethyl benzidine, and 0.0003% hydrogen peroxide (Sigma) was added for 30 min. Color development was measured at 450 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA). To measure the release of the ectodomain of NOTCH3 into conditioned medium, an ELISA was developed that relies on HRP-linked antibodies specific for non-overlapping epitopes in NOTCH3-EGF repeat region.

Compounds—The metalloprotease inhibitor GM6001 was purchased from Chemicon (Temecula, CA). The γ-secretase inhibitor compound E was purchased from Calbiochem.

Western Blot Analyses—Polypeptides in whole cell lysates were resolved by SDS-PAGE. NOTCH3 polypeptides were detected on blots with a polyclonal antibody specific for the C terminus of the intracellular domain (OriGen, San Diego, CA) or NOTCH3 mAbs A2 and A13.

Luciferase Reporter Assays—293T-NOTCH3 cells stably transfected with a Notch-sensitive-luciferase reporter gene plasmid were co-cultivated with control cells or 293T-JAGGED1 cells at 1:1 ratio or cultured on plates coated with JAGGED1-IgG Fc fusion protein or DLL4 (R&D Systems, Minneapolis, MN), for 28–40 h. The Notch-sensitive luciferase reporter gene used contains eight tandem repeats of the CSL-consensus binding motif positioned 5′ of the firefly luciferase gene in the pTA-Luc vector (Clontech, Mountainview, CA). Luciferase reporter activities were assayed using the Stable Luciferase Assay kit following the manufacturer’s protocol (Promega, Madison, WI) and a TD-20 luminometer (Turner Design, Sunnyvale, CA).

To determine the specificity of activating and inhibitory antibodies, U2OS Tet-on flp-in cells bearing isogenic transgenes encoding NOTCH1-, NOTCH2-, or NOTCH3-Gal4 chimeric receptors were transfected with a 5 μg of Gal4-firefly luciferase and 100 ng of pRL-TK-RENilla luciferase reporter plasmids. After 24 h, the cells were split onto OP9 or OP9-DLL1 feeder cells in 24-well plates in the presence of tetracycline (1 μg/ml) and control or NOTCH3 mAbs (10 μg/ml). Following 24 h of co-culture, luciferase activities were measured in whole cell lysates using a dual luciferase kit (Promega) and a Turner
NOTCH3 Antibodies and Their Implications for Notch Regulation

TD-20 luminometer. Normalized luciferase activities for each well were expressed as the ratio of firefly luciferase to Renilla luciferase.

RNA Extraction and QRT-PCR—Extraction of total RNA from cell lines or tumor samples was done using the RNeasy Mini Kit (Qiagen, Valencia, CA). Quantitative real-time PCR (QRT-PCR) was performed in duplicate with the ABI Prism 7900 (Applied Biosystems, Foster City, CA) sequence detection system and TaqMan reagents according to the manufacturer’s instructions.

Epitope Mapping—Soluble Notch-Fc fusion protein expression constructs were generated by PCR. Domain and subdomain swaps between NOTCH3 and NOTCH1 were carried out based on sequence alignments performed using LaserGene DNAStar (Madison, WI). cDNAs were assembled in pcDNA3.1-Hyg. Polypeptides were produced by transfection of pcDNA3.1 plasmids into Chinese hamster ovary cells with Lipofectamine 2000 (Invitrogen). After culture for 2–3 days in Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum, conditioned media were collected, cleared by centrifugation, and subjected to analysis by ELISA as described above. The amounts of various polypeptides recovered from conditioned media were assessed semi-quantitatively by ELISAs performed with an antibody specific for human IgG Fc. Binding results for each antibody to individual polypeptides were expressed relative to binding of the same antibody to the NRR region of NOTCH3 (which was considered 100% binding activity).

Antibody Competition Assay—Immunol II (Dynatech Laboratories, Chantilly, VA) microtest plates were coated overnight with a NOTCH3 NRR-Fc fusion polypeptide at 0.2 μg/ml in PBS (100 μl/well). After blockade of nonspecific binding sites with 200 μl of 2% bovine serum albumin in PBS (PBS-B buffer), the wells were washed with PBS-T buffer (PBS containing 0.05% Tween 20). A 1:20,000 dilution of HRP-conjugated monoclonal antibody was then mixed with a dilution series of various competitor antibodies in “U” bottom 96-well plates. After 15 min at room temperature, 100 μl/well of these mixtures was transferred to the ELISA plate wells for 1 h. The wells were then washed with PBS-T, and the amount of HRP-labeled mAb bound to the NOTCH3 LNR-HD domain-Fc fusion polypeptide was determined by ELISA.

Structural Modeling—The homology model of the human NOTCH3 NRR (residues 1387–1631) was generated with the Swiss Model web interface (38), using the coordinates of the human NOTCH2 NRR (pdb code: 2O04) as an input model. The input NOTCH3 sequence used for modeling lacked the non-conserved loop containing the furin cleavage site, because this unstructured region was excised from the crystallized NOTCH2 NRR (39). The first approach mode was used to generate an initial homology model and accompanying sequence alignment, which was then manually modified to account for residues absent from the initial model. These additional residues were added to the C terminus of the model coordinates using Coot (40). A final correction of the alignment was then performed in Swiss Pdb Viewer. This model and the accompanying corrected sequence alignment were then input into the Optimization Mode of Swiss Model. The resultant pdb file was used to display the antibody epitope locations.

Apoptosis and Proliferation Assay—293T-NOTCH3 cells were seeded in IgG Fc- or JAGGED1-Fc-coated 96-well plates in the presence of different antibodies in serum-free Dulbecco’s modified Eagle’s medium for 24 h. Cells with active caspase 3 were detected by staining with NucViewTM 488-conjugated DEVD (Biotium, Inc., Hayward, CA) followed by flow cytometry. To assess proliferation, 3 × 10^4 293T-NOTCH3 cells were seeded in the presence of different antibodies in 96-well plates coated with JAGGED1-Fc or IgG Fc. After growth for 24–48 h in Dulbecco’s modified Eagle’s medium containing 7% fetal calf serum, viable cells were enumerated under the microscope by trypan blue exclusion.

Migration Assay—293T-NOTCH3 cell migration assays were carried out in 24-well Transwell plates (Corning, Acton, MA). Insert membranes (average pore size, 8 μm) were coated with JAGGED1-Fc or IgG Fc or mock treated on the upper surface, and 10% fetal bovine serum medium was added to the lower chamber as a chemoattractant. Cells were seeded on the upper surface in the presence of different antibodies. Cell migration to the lower chamber was determined by counting under the microscope.

Sphere Formation Assay—The 293T-NOTCH3 cells were cultured in 96-well plate coated with IgG Fc or JAGGED1-Fc in the presence of different antibodies in serum-free medium. Cell morphology was documented after 15–20 h. Sphere formation was quantified by wash-off experiments, in which the floating spheres were removed by aspiration of the medium. The remaining cells were stained with crystal violet in 40% methanol and 60% PBS for 15 min. After washing three times with deionized water, the dye retained in the cells was extracted with 30% acetic acid and measured by absorption at 590 nm.

RESULTS
Identification and Characterization of Inhibitory and Activating NOTCH3 mAbs—To perform an unbiased search for functional NOTCH3 antibodies, we generated mAbs against two NOTCH3 polypeptides consisting of (i) the EGF repeat region or (ii) the NRR, which together span the entire NOTCH3 ectodomain. NOTCH3 mAbs were first identified using ELISA and high throughput fluorescence cell staining assays (FMAT™), and then tested for functional effects in cell-based assays using a Notch-sensitive luciferase reporter gene. Both strong inhibitory and activating antibodies were identified, all of which were raised against the NRR (summarized in Fig. 1A).

The two most potent antagonist mAbs were designated 256A-4 (A4) and 256A-8 (A8). At doses of 0.1–0.3 μg/ml, A4 and A8 completely inhibited the activation of NOTCH3 by multiple DSL ligands, including cell-associated JAGGED1 (Fig. 1B), JAGGED2 (supplemental Fig. S1A) and DLL1 (Fig. 2C), and soluble DLL4 (supplemental Fig. S1B) and JAGGED1 adsorbed to the surface of culture dishes (supplemental Fig. S1C). A4 and A8 also inhibited JAGGED1-induced up-regulation of HES5 and HHEY2, two well characterized Notch genes, in 293T cells overexpressing NOTCH3 (293T-NOTCH3 cells) (Fig. 1C). In addition, A4 and A8 did not affect the binding of soluble ligand to NOTCH3 (supplemental Fig. S1D); data not
shown), indicating that inhibition by these mAbs does not involve competition with ligand for access to ligand-binding sites.

As anticipated, we also identified antibodies specific for the EGF repeat region that inhibited NOTCH3 activation, perhaps through competition for ligand binding. However, in direct comparisons, mAbs A4 and A8 were substantially more potent than the most active mAbs (A45 and A79) raised against the EGF repeat region, which were only weak antagonists of JAGGED1- and DLL4-induced NOTCH3 signaling (Fig. 1A and supplemental Fig. S3, A and B), and completely inactive against JAGGED2-induced NOTCH3 signaling (Fig. 1A and supplemental Fig. S3C).

Among mammalian Notch receptors (NOTCH1–4), NOTCH1 and NOTCH2 have the highest homology to NOTCH3 within the NRR region. To establish the specificity of A4 and A8, we tested their activities against isogenic cell lines that express NOTCH1-, NOTCH2-, or NOTCH3-Gal4 chimeric receptors (Fig. 2A). In response to DSL ligands, these receptors stimulate Gal4-luciferase reporter gene activity in a γ-secretase-dependent fashion (data not shown). A4 and A8 inhibited the DLL1-induced activation of the NOTCH3-Gal4 receptor, while having minimal effects on the activation of the NOTCH1- and NOTCH2-Gal4 receptors (Fig. 2C), demonstrating that these antibodies are specific NOTCH3 antagonists.

We also identified four mAbs that activated NOTCH3 signaling in the absence of ligand (Figs. 1D and 2D; data not shown). The most potent agonist antibody 256A-13 (A13) was characterized further. A13 strongly activated the chimeric NOTCH3-Gal4 receptor but did not affect the NOTCH1- or NOTCH2-Gal4 chimeric receptors (Fig. 2D). Treatment of cells expressing NOTCH3 with mAb A13 resulted in increased shedding of NOTCH3 ectodomains into conditioned medium in a manner that was sensitive to the metalloprotease inhibitor GM6001 (Fig. 3A). In addition, mAb A13 stimulated the cleavage of NOTCH3 at site S2, based on the accumulation of NTM* (the product of S2 cleavage) in the presence of a γ-secretase inhibitor (which stabilizes NTM*). Notably, A13 was more effective at inducing the accumulation of NTM* than immobilized JAGGED1 (Fig. 3B). In contrast, mAb A4 and A8 inhibited the JAGGED1-induced metalloprotease cleavage of NOTCH3 (Fig. 3B), consistent with their antagonistic effects on reporter and target gene activation. As expected, the stimulation of reporter gene activity by A13 was completely abrogated by a γ-secretase inhibitor (data not shown). A13 also stimulated the cleavage and activation of a form of NOTCH3 that lacks the ligand bind-
NOTCH3 Antibodies and Their Implications for Notch Regulation

FIGURE 2. Specificity of NOTCH3 modulatory antibodies. A, structure of chimeric NOTCH-Gal4 fusion receptors used to assess specificity. The schematic shows the structures of the intact NOTCH1 receptor (N1) and the chimeric NOTCH1-, NOTCH2-, and NOTCH3-Gal4 receptors used in these assays. EGF, EGF-like repeats; L, LNR repeats; D1, N-terminal portion of the heterodimerization domain; D2, C-terminal portion of the heterodimerization domain; R, RAM domain; ANK, ankyrin repeat domain; TAD, transcriptional activation domain; P, PEST domain; Nc, Notch extracellular subunit; Nm, Notch transmembrane subunit. B, tetracycline-dependent expression of Notch-Gal4 chimeric receptors. A Western blot containing whole cell lysates prepared from the indicated U2OS cell lines after growth for 24 h in the presence of carrier (ethanol, –) or of tetracycline (1 μg/ml, +) is shown. The blot was stained with polyclonal rabbit antibody that is specific for the TAD of NOTCH1 (46). Nc-Gal4, unprocessed full-length Notch-Gal4 fusion receptors; Nm-Gal4, transmembrane subunits of mature Notch-Gal4 receptors created by processing at site S1. C, effect of mAbs A4 and A8 on DLL1-induced Gal4-luciferase reporter gene activity in U2OS cells stably expressing NOTCH1-, NOTCH2-, or NOTCH3-Gal4 chimeric receptors. Luciferase assays were performed on lysates prepared after 24 h of co-cultivation with either OP9 or OP9-DLL1 feeder cells in the presence of control or NOTCH3 antibodies (10 μg/ml). NOTCH1-Gal4 control cells co-cultivated with OP9 cells had the lowest normalized luciferase activity, which is arbitrarily set to 1; all other luciferase activities are expressed relative to this control value. D, effect of mAb A13 on luciferase reporter gene activity in U2OS cells stably expressing NOTCH1-, NOTCH2-, or NOTCH3-Gal4 chimeric receptors. Luciferase assays were performed on lysates prepared after 24 h of treatment with either mAb A13 or isotype-matched control antibody (G3) at concentrations of 10 μg/ml. NOTCH1-Gal4 cells treated with the control antibody had the lowest normalized luciferase activity, which is arbitrarily set to 1; all other luciferase activities are expressed relative to this control value.
autoinhibition either by directly prying the structure open or by triggering an allosteric conformational change.

Inhibitory and Activating Antibodies Have Opposite Effects on NOTCH3-mediated Cellular Changes

Overexpression of NOTCH3 in the absence of ligand had little effect on the behavior of 293T cells, based on comparisons to parental cells or cells transfected with empty vector (data not shown). However, when NOTCH3 overexpressing 293T cells (293T-NOTCH3...
Table 1
Summary of epitope-mapping through subdomain and amino acid cluster swaps within the NOTCH3 LNR1 (L1)

<table>
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<th>Expression constructs</th>
<th>Wild-type and swapped sequences of NOTCH3 L1 domain</th>
<th>A4</th>
<th>A8</th>
<th>A13</th>
<th>A2</th>
<th>G3</th>
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<tr>
<td>NOTCH3-L1</td>
<td>EPFRCPRAACQAKRDQDRDCRECNSPCGRHDGIDCSLSVGG</td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>L1-sub1</td>
<td>EECELPE</td>
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<td>+++</td>
<td>+++</td>
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<tr>
<td>L1-sub2</td>
<td>EAGMKVS</td>
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<td>L1-aa swap1</td>
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<tr>
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Table 2
Summary of epitope-mapping through subdomain and amino acid cluster swaps within the NOTCH3 HD2 (D2)

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<td>+++</td>
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<tr>
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Figure 5. Homology model of the human NOTCH3 NRR, derived from the crystal structure of the human NOTCH2 NRR (pdb: 2004) using the Swiss Model web interface (see “Experimental Procedures”). LNRs 1–3 are colored light pink and the HD domain is light cyan. A, ribbon representation of the model. Residues implicated in antibody binding by epitope mapping studies are shown in stick representation. Residues in L1 that bind inhibitory mAbs A4 and A8 only are hot pink; residues in L1 that bind both blocking antibodies and activating mAbs A13 are purple; residues in D2 that bind only A4 and A8 are teal. B, surface representation. Top: deduced epitope for A4 and A8, which includes sites in L1 (hot pink) and D2 (teal). Bottom: deduced epitope for A13 in L1 (purple).

Our studies demonstrate that it is possible to develop potent antibodies that selectively inhibit or induce activating proteolytic cleavage of individual mammalian Notch receptors. This “proof-of-principle” demonstration has significant mechanistic and applied implications.

The small number of the antibodies analyzed in our unbiased functional screens that were potent and specific modulators of NOTCH3 S2 cleavage all bind to epitopes on one face of the NRR (also called the LNR/HD region), reinforcing the notion that this Notch-specific structural domain is the key to understanding how the activation of these receptors is normally reg-
The inhibitory activity of antibodies that clamp the LNR and HD domains together is in line with predictions drawn from the structure of autoinhibited NOTCH2 (39), which suggest that the movement of the LNRs away from the HD domain is a necessary prerequisite for exposure of the S2 cleavage site, although it is still unclear if this involves a simple conformational change or the physical dissociation of the heterodimeric subunits. It will be of interest to determine how antibodies that bind LNR1, such as mAb A13, expose the S2 site, and whether the mechanism of antibody-induced S2 cleavage precisely recapitulates events associated with ligand-induced S2 cleavage, or occurs through a non-physiologic allosteric effect (e.g. as is the case for activating integrin antibodies (42)). More generally, the observation that all of the strong modulatory antibodies identified bind the NRR will serve to focus further efforts to develop potent and selective antibody and small molecule modulators of human Notch receptors.

The NOTCH3-selective mAbs should be valuable tools in understanding the role of this receptor in various disease processes. Increased NOTCH3 activity has been implicated in experimental leukemias (23, 24) and sporadic human ovarian and lung carcinomas (25, 26), yet the role of individual Notch receptors in cancers lacking acquired gain-of-function mutations (which have thus far only been identified in human T-cell acute lymphoblastic leukemia) has been uncertain. The availability of selective NOTCH3 inhibitors and agonists should clarify the specific role of NOTCH3 in these and other cancers. It is also possible that these antibodies will be of utility in investigating the role of NOTCH3 in other disease states, such as CADASIL (18), a vasculopathy caused by inherited mutations in NOTCH3, and disorders of the immune system (43).

In principle, it should be possible to develop antibodies that selectively modulate the activities of NOTCH1, NOTCH2, and NOTCH4 as well. Although knock-out mice have clarified certain non-redundant Notch functions, most cell types express multiple Notch receptors. The capacity to activate and/or inhibit specific Notch receptors, either individually or in combination, would allow their functions to be further resolved and enable understanding of their functional interrelationships, particularly in disease settings. This interplay may take the form of competition among ICNs for access to promoters that contain monomeric CSL response elements and direct interaction between ICNs (e.g. ICN1 and ICN3) in higher order het-

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**FIGURE 6.** Inhibitory and activating antibodies have opposite effects on NOTCH3 mediated cellular changes. A, effects on cell proliferation. 293T-NOTCH3 cells were seeded in 96-well plates coated with JAGGED1-Fc or IgG in the presence of activating mAb A13 (0.01 μg/ml) or IgG Fc in the absence of serum. Activated caspase-3 was detected by treatment with fluorescently labeled DEVD followed by flow cytometry. C, effects on cell migration. 293T-NOTCH3 cells were cultured in Transwell plates coated with IgG Fc or JAGGED1-Fc in the presence of different mAbs (10 μg/ml). Migration of cells to the bottom of the well was quantified by counting under a microscope; representative fields are shown. All data presented are mean values (n = 3); error bars correspond to standard deviations. D, effects on JAGGED1-induced sphere formation. 293T-NOTCH3 cells were cultured in 96-well plates coated with IgG Fc or JAGGED1-Fc. Antibodies were added to a final concentration of 10 μg/ml. Cells were photographed after 20–24 h. The bar graph shows results from a wash-off experiment, in which the cells remaining after washing were stained with crystal violet and quantified by absorption at 590 nm.
NOTCH3 Antibodies and Their Implications for Notch Regulation

erodimeric Notch transcriptional activation complexes, which may form on target gene promoters containing paired CSL-binding elements (44, 45). In addition, soluble antibody agonists may help to expand or regulate the ex vivo differentiation of certain cell types (e.g. stem cells) for purposes of downstream experimental or therapeutic use. It is also likely that antibody inhibitors will mitigate toxicities associated with γ-secretase inhibitors, such as secretory diarrhea (29). Further development and characterization of antibody modulators of Notch activity is thus likely to have broad experimental and therapeutic impact.

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