Notch Signaling Enhances Nestin Expression in Gliomas[1]*

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Abstract

Recent findings suggest that Notch signaling is active in brain tumors and stem cells, and that stem cells or cells with progenitor characteristics contribute to brain tumor formation. These stem cells are marked by expression of several markers, including nestin, an intermediate filament protein. We have studied how the Notch signaling pathway affects nestin expression in brain tumors. We find that Notch receptors and ligands are expressed *in vitro* and in human samples of glioblastomas, the highest grade of malignant gliomas. In culture, Notch activity activates the nestin promoter. Activation of the Notch pathway also occurs in a glioblastoma multiforme mouse model induced by Kras, with translational regulation playing a role in Notch expression. Combined activation of Notch and Kras in wild-type nestin-expressing cells leads to their expansion within the subventricular zone and retention of proliferation and nestin expression. However, activation of Notch alone is unable to induce this cellular expansion. These data suggest that Notch may have a contributing role in the stem-like character of glioma cells.

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Introduction

Among tumors of the central nervous system (CNS), glioblastoma multiformes (GBMs) are the most aggressive tumors with the poorest clinical prognosis. These tumors consist of cells that are astrocyte-like but have complex genetic makeup and expression patterns. One phenomenon that can contribute to this complexity is the presence of stem-like cells within the tumor and the activation of pathways that control cellular differentiation. Several groups have been able to identify unique populations within GBMs with stem cell properties [1–3]. However, which pathways contribute to the formation of stem-like character and to the dysregulation of differentiation remain to be fully described. One marker that has been used to identify neural stem cells is the intermediate filament protein nestin [4]. Nestin-expressing cells have the ability to differentiate into multiple lineages, including neurons and glial cells within the CNS—a characteristic found in glioma stem cells [5].

In trying to understand signaling events that regulate GBMs, work in our laboratory has previously identified the Ras and Akt pathways as being elevated in human GBMs, with a unique synergistic effect on the translation of a subset of mRNA [6]. One such target of increased translation is Notch1 mRNA. Notch (Notch1–4 in mammals) is a family of transmembrane receptors that regulate cell–cell signaling (reviewed in Artavanis-Tsakonas et al. [7]). Notch ligands (Delta-like1, Delta-like3, and Delta-like4, and Jagged1–2 in mammals) are also transmembrane proteins and, when bound to Notch, act to expose the receptor to proteolytic activation. Presenilins cleave Notch to generate a Notch1 intracellular domain (NICD), which then translocates to the nucleus to act as a transcriptional activator [8].

Activated Notch signaling appears particularly capable of affecting both tumorigenesis and stem cell development. Notch signaling was first shown to be inhibitory toward neurogenesis and essential in maintaining a pool of undifferentiated stem cells [9,10]. Deletion of *Notch1* results in reduction in neural stem cells; in *presenilin*$^{-/-}$ mutants defective in Notch signaling, neural stem cells have reduced proliferative capacities [11]. Later studies have expanded the function of Notch to involve important cell fate decisions throughout glial and neuronal development. In the oligodendrocyte lineage, Notch activation has been shown to suppress terminal oligodendrocyte differentiation but also to support the specification of oligodendrocyte precursors from the initial stem cell pool [12,13]. Notch signaling can promote the specification of Muller glia in the rat retina [14,15], radial glia in the telencephalon from cortical stem cells [16], and astrocytes in the adult brain from hippocampal multipotent progenitors [17].

Notch signaling has been implicated in various steps throughout tumorigenesis. Studies have identified the Ras pathway, in particular, as being able to collaborate with the Notch pathway to maintain and establish neoplastic phenotype [18,19]. In CNS tumors, Notch signaling components were found to be deregulated in meningiomas [20], and its activity is

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observed to be critical in medulloblastomas [21,22]. In gliomas, Notch1 and its ligands Delta-like1, Delta-like3, and Jagged1 have also been shown to be involved in glioma cell survival, differentiation, and proliferation [23,24].

In this study, we investigated the role of Notch activation in glioblastoma biology. We confirmed that Notch receptors and ligands are expressed in human GBMs. This was also true in a Kras-induced mouse GBM model. In these tumors, Notch may participate in the transcription of specific gene targets, particularly that of nestin, a neural progenitor marker. The activation of nestin promoter by Notch was also seen in cultures where Notch can directly act on the nestin-regulatory region to activate its transcription. *In vivo*, we found that combined activation of Notch and Kras signaling was sufficient to generate lesions along the subventricular zone (SVZ). These lesions expressed nestin and a marker of proliferation, suggesting that they may be early precursor lesions to tumorigenesis.

Materials and Methods

Tumor Samples

All human tissues were collected by the Memorial Sloan-Kettering Institute tissue bank, snap-frozen, and stored at −80°C. Samples in liquid nitrogen were ground in mortar and pestle. Protein was extracted from the powder through lysis with T-per tissue extraction solution (Pierce Biochemical, Rockford, IL) supplemented with miniTab protease inhibitors (Boehringer Mannheim, Ingelheim, Germany), 30 mM sodium fluoride, and 1 mM sodium vanadate.

Microarray

Mouse tumors and $arf^{-/-}$ cortex were dissected from brains and frozen in liquid nitrogen. Tissue in liquid nitrogen was ground to powder form by mortar and pestle. Probe preparation and hybridization to chips were performed as described before [6]. Briefly, RNA was prepared using Trizol (Invitrogen, Carlsbad, CA) and Qiagen RNA Easy kit (Qiagen, Valencia, CA). cRNA was generated by *in vitro* transcription using standard procedures. Probes were hybridized to Affymetrics MOE430 chip (Affymetrics, Santa Clara, CA). Chips were scanned by a Hewlett Packard GeneArray Scanner (Hewlett Packard, Palo Alto, CA), and data were collected by MicroArray Suite Software (Affymetrics). 5′ Upstream promoter sequences were derived from ensembl.org.

Western Blot Analysis

Proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blocking reagent was 5% nonfat dry milk in phosphate-buffered saline (PBS)–0.1% Tween 20. Primary and secondary antibodies were prepared in the same blocking reagent. Horseradish peroxidase (HRP)–conjugated secondary antibodies were used at 1:1000 dilution and visualized using ECL chemiluminescence (Amersham, Piscataway, NJ), hNotch1 1:50 [bTAN20; Developmental Studies Hybridoma Bank (DSHB) (Iowa City, IA)], hNotch2 1:50 (C651.6DbHN; DSHB), mNotch1 1:500 (Neomarkers, Fremont, CA), Delta-like1 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA), Jagged1 1:500 (Santa Cruz Biotechnology), actin 1:1000 (Santa Cruz Biotechnology), anti-mouse HRP (Boehringer Mannheim), and anti-rabbit HRP (Amersham).

DNA Plasmids

The construction of the retroviral vectors RCAS-Kras and RCAS-Akt has been previously described [25,26]. The RCAS(B) mouse Notch1 ICD plasmid was provided by Dr. David Anderson and subcloned into RCAS(A). The RCAS(B) control virus consists of noncoding (empty) or nonproductive virus plasmids. Rat Delta1 and rat Jagged1 cDNA were provided by Dr. Gerry Weinmaster. The nestin-tk reporter plasmid was provided by Dr. Urban Lendahl [27,28].

Infection of tv-a Transgenic Mice and Tissue Processing

Generation of the transgenic mouse line that expresses the RCAS receptor from the nestin promoter nestin tv-a (Ntv-a) and Ntv-a $arf^{-/-}$ mice has been previously described [25,29]. Neonatal mice were injected in the cerebral hemisphere with DF-1 chicken fibroblasts, producing appropriate RCAS viruses on postnatal day 1. Mice were monitored and then sacrificed when symptomatic or at the end of the study. Brains were fixed in formalin, processed, embedded in paraffin, and sectioned as previously described [30]. Chi-square analysis was used to determine statistical significance.

In Situ *Hybridization*

Probe fragments were cloned into pDrive Cloning Vector (Qiagen) using polymerase chain reaction (PCR) or restriction digest. Probe lengths correspond to the following stretches of cDNA, as demarcated by the following 5′ and 3′ sequences:

Notch probe: 5′seq CAGCATGGCCAGCTCTGGTT, 3′seq AGCAGCATCCACATTGTTCA
Hes1 probe: 5′seq ATGCCAGCTGATATAATGGA, 3′seq TCAGTTCCGCCACGGCCTCC
rDelta1 probe: 5′seq ATCACACCTGGAGCCGAGAG, 3′seq GGCCGCTACTGTGAAGGTCC
rJagged1 probe: 5′seq GGCCGGGGCGCCCCTTGAGC, 3′seq GGCTGGGGTTTATCATGCCT.

Radioactive sense and antisense probes incorporating [^{32}P]UTP were prepared using T7 or SP6 polymerase. Tissue sections were deparaffinized and treated with proteinase K. Slides were prehybridized with salmon sperm DNA and hybridized overnight with probe in hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris pH 8.0, 5 mM EDTA, 10 mM Na-phosphate, 10% dextran sulfate, 1× Denhardt's solution, and 500 μg/ml yeast tRNA) at 65°C. Slides were washed, dipped in emulsion, exposed for 2 to 4 weeks, and developed. Three to five tumors of each type were analyzed with each probe set.

Image Analysis

For signal density analysis, paired dark-field images of normal cortex and tumor regions were taken from the same

slide using Zeiss Axioplan microscopes (Zeiss, Oberkochen, Germany). All images were taken at fixed exposure and resolution. Ten such paired images were generated for each tumor type. Images were then processed on Metamorph software (Metamorph, Sunnyvale, CA) to determine the area of positive signal pixels. Areas of paired images were compared (tumor to normal tissue), and statistical significance was determined using *t*-test.

Immunohistochemistry (IHC)

Sections were processed for IHC as described previously, with antigen retrieval in citrate buffer with steam [31]. Antibody detection was visualized with peroxidase and DAB processing (Vector, Burlingame, CA). The antibodies used are as follows: 9E10 1:200 (human c-myc; Covance, Princeton, NJ), nestin 1:1000 (PharMingen, San Diego, CA), and proliferating cell nuclear antigen (PCNA) 1:1000 (Chemicon, Temecula, CA).

Luciferase Assay

Cells were transfected with plasmid containing pHES1-luciferase, pCMV-RL (Promega, Madison, WI), and RCAS expression vector (control, Notch, or Delta) using Fugene (Roche, Nutley, NJ) according to the manufacturer's instructions. Lysates were prepared, and luminescence was determined according to the Promega Dual-Luciferase Reporter System protocol (Promega). Readings were performed with a Turner Biosystems platereader. Firefly luminescence was normalized to renilla luminescence. Hes1 reporter was subjected to PCR with genomic DNA using the primers 5′seq ACGGGGTACCCTCAGGCGCGCGCCATTGGCC and 3′seq CCGAAGATCTGCTTACGTCCTTTTACTTGAC. The fragment contains suspected CBF-1–binding sites. The fragment was cloned into the PGL3-Basic Vector (Promega) using *Kpn*I and *Bgl*II.

β-Galactosidase Assay

Cells were transfected with β-galactosidase reporter constructs using Fugene according to manufacturer's instructions. Two days after transfection, cells were fixed in neutral-buffered formalin and washed in PBS. Cells were then incubated at 37°C with a solution containing 1 mg/ml X-gal, 20 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$ for 6 hours until a blue precipitate was visible.

Results

Presence of Notch Receptors and Ligands in Primary Human Glioblastomas

To determine whether Notch signaling could contribute to gliomas and GBMs in particular, we compared astrocytic mixed astrocytic gliomas (World Health Organization grades II and III), GBM expression profiles, and protein levels. Previous work in our laboratory has described a microarray experiment that identified genes overexpressed in human gliomas [32]. This experiment compared 9 GBMs and 10 non-GBM gliomas to pooled normal brain. Using this database, we found that five of nine GBMs had a ≥ 2-fold expression in Jagged1 mRNA compared to normal brain, whereas none of the non-GBM gliomas had any appreciable difference in Jagged1 expression (Figure 1*A*). Unfortunately, the probes for Notch1 and Delta-like1 were not represented on the array used at the time. This suggested that Notch ligand expression may contribute to glioblastoma formation.

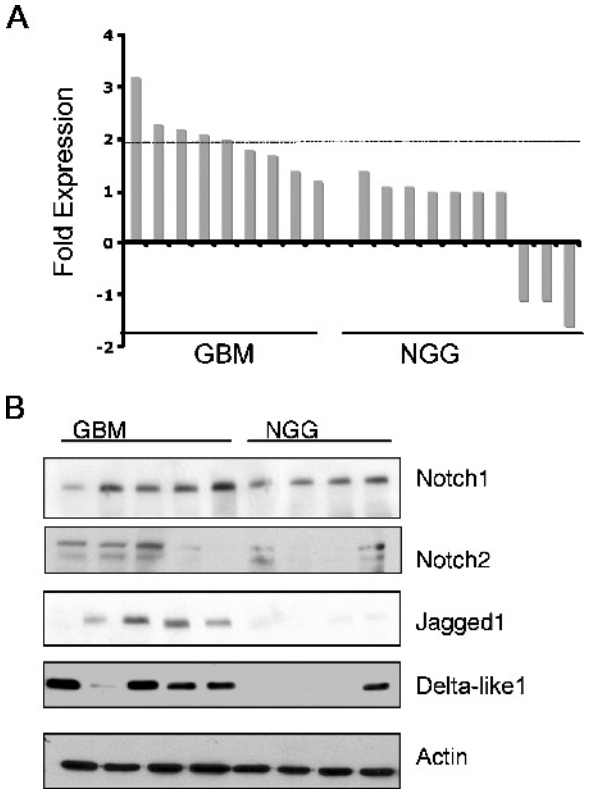

Figure 1. *Notch receptor and ligand expression in human gliomas. (A) Analysis of Jagged1 mRNA expression in glioblastomas (GBMs) and non-GBM gliomas (NGGs) compared to normal brain. Data were derived from the microarray of Tanwar et al. [32]. (B) Western blot analysis of Notch receptors 1 and 2, ligands Delta-like1 and Jagged1, and corresponding actin bands in glial tumors.*

To determine the protein levels of Notch receptors and ligands in human tumors, a selection of primary human tumors (GBMs and non-GBM gliomas) was subjected to Western blot analysis (Figure 1*B*). We found Notch1 to be expressed in all tumor types examined, whereas Notch2 was expressed in a subset of tumors. Notch ligands Delta-like1 and Jagged1 were more selectively expressed in GBMs. The coexpression of Notch and its ligands demonstrates that autocrine or juxtacrine modes of activation are possible, particularly in GBMs and but perhaps also in a smaller subset of glial tumors.

Expression of Notch Receptors and Ligands in Mouse Models of Tumors

Given the finding in human tumors, we investigated whether this pattern of expression was also found in mouse models of glial tumors, particularly comparing mouse models of GBMs and oligodendrogliomas, a clinically less aggressive form of glioma. In addition, because we know that translation

may play an important role in GBMs, we assessed differences in expression in both mRNA and protein levels. Previously, we have developed various mouse models of CNS tumors generated through the RCAS/tv-a system. In this system, tv-a receptor is expressed from a selective promoter by a transgene, whereas an RCAS virus delivers oncogenes only into these targeted cells [33]. Two tv-a transgenic lines have been used in glioma modeling studies. One expresses tv-a from the nestin promoter (Ntv-a), whereas the other expresses tv-a from GFAP promoter (Gtv-a). The cell specificity of gene transfer mediated by RCAS infection in these mice is determined by promoter-driven tv-a expression. After infection, oncogenes are driven from the RCAS viral promoter and are unaffected by the activity of the promoter driving the tv-a transgene. The models include platelet-derived growth factor B (PDGFB)-induced oligodendrogliomas in wild-type mice and Kras-induced spindle gliomas and GBMs in *arf*-null mice [29,30]. Of these tumors, the Ras and Akt pathways are upregulated only in Kras-induced tumors and not in PDGFB oligodendrogliomas [31]. Thus, the Kras-induced model more closely resembles human GBMs and has the potential to exhibit similar translation effects. To investigate the expression of Notch pathway components, *in situ* and Western blot analyses were performed on these different glioma subtypes.

It has been previously reported that Notch1 is expressed in selected cells within the cerebellum in adult rat brain, specially in the Purkinje cell layer [34]. We have confirmed that we could obtain a similar pattern using our antisense probe and had little background with the corresponding sense probe control (Figure 2, *A* and *B*). We analyzed tumors driven from Ntv-a mice where the cell of origin was nestin-expressing. On *in situ* hybridization analysis, Notch1 mRNA is overexpressed in both Ntv-a PDGF and Ntv-a Kras $arf^{-/-}$ tumors compared to normal brain (Figure 2*C*). Because translation may regulate Notch1 expression, defining relative mRNA and protein levels may be important in understanding Notch expression levels. To assess potential translation effect, we semiquantitatively determined relative mRNA expression levels by comparing *in situ* signal density in tumor regions to control normal brain regions within the same section (Figure 3*A*). Images were processed by Metamorph software to determine the total positive signal image area in each image. Tumor regions were then compared to normal brain regions from the same slide. Kras-induced tumors had

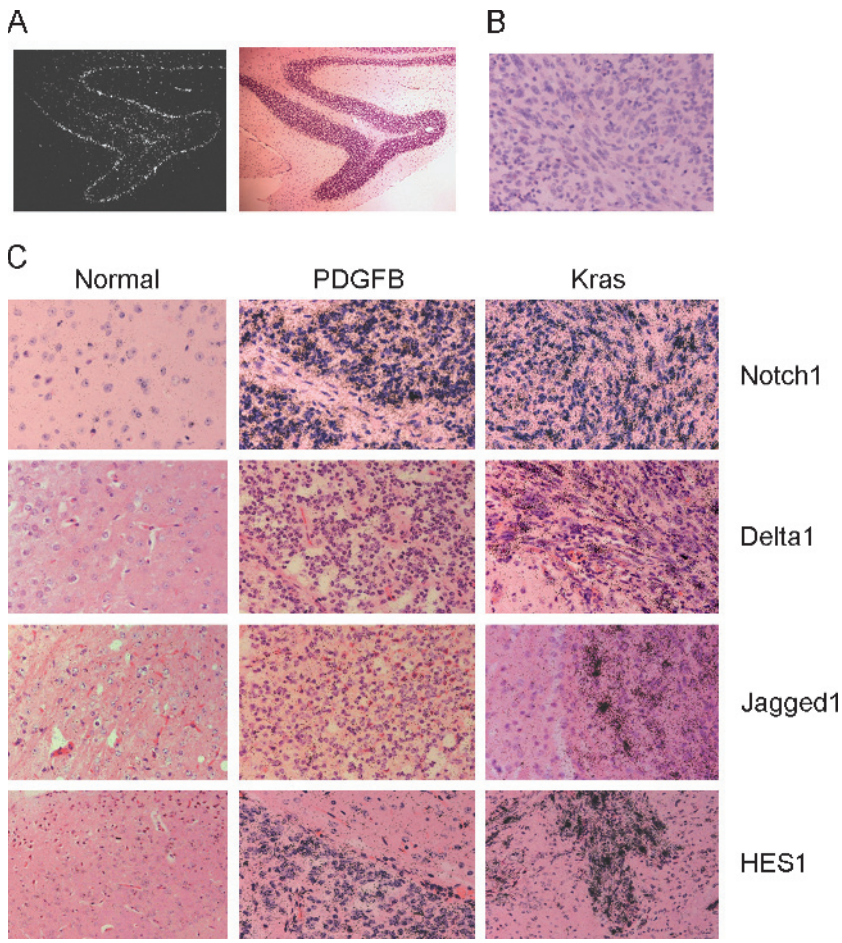

Figure 2. *Notch receptor and ligand expression in mouse glioma models. (A) Dark-field image of antisense Notch1* in situ *hybridization, and hematoxylin and eosin (H&E) image of the same section in the cerebellum. Positive signal corresponds to a previously described pattern for Notch1 [34]. (B) Notch1 sense strand* in situ *hybridization control in tumor tissues. (C) Antisense* in situ *expression of Notch1, Delta-like1, Jagged1, and Hes1 mRNA in normal brain; PDGFB-induced oligodendroglioma; and Kras-induced spindle GBMs.*

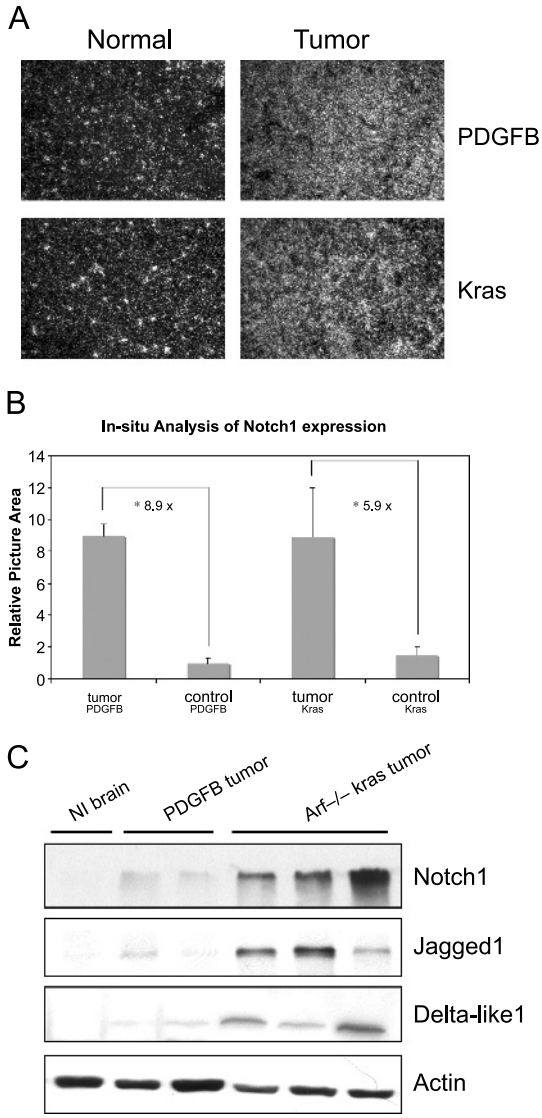

Figure 3. *Semiquantitative analysis of Notch1 mRNA and protein in PDGFB and Kras tumors. (A) Sample dark-field images of Notch1* in situ *in normal and tumor regions of PDGFB and Kras tumors. (B) Image analysis of positive signal regions in tumors. Positive pixel area (white grains in A) normalized to positive pixel areas in control normal brain images (*P < .01). (C) Western blot analysis of Notch1 and ligand expressions in PDGFB-induced oligodendrogliomas and Kras-induced spindle GBMs.*

a 5.9-fold higher level of Notch1 signal compared to controls, whereas PDGFB-induced oligodendrogliomas had an 8.9-fold higher level (Figure 3*B*). Expression of Notch1 protein was also found to be higher in both tumor types compared to normal brain (Figure 3*C*). However, Notch1 protein levels were much higher in Kras GBMs compared to oligodendrogliomas even though *in situ* analysis revealed that mRNA levels were relatively lower in Kras GBMs than in PDGF oligodendrogliomas compared to normal brain (5.9-fold *vs* 8.9-fold). This supports our previous finding that Ras and Akt signaling pathways may selectively recruit Notch1 mRNA into polyribosomes to be translated into protein [6]. Substantially more active Akt and Ras signaling pathways are found in Kras-induced GBMs compared to PDGFB-induced oligodendrogliomas and may explain why Notch1 protein levels are higher in our mouse models of these tumors.

Delta-like1 and Jagged1, ligands of Notch receptors, were found to be overexpressed by both *in situ* and Western blot analyses in Kras-induced tumors (Figures 2*C* and 3*C*). In PDGFB-induced oligodendrogliomas, these ligands are either not elevated or only mildly elevated (Figures 2*C* and 3*C*). One common transcriptional target of Notch signaling is HES1. We tested for HES1 expression in our mouse tumor models and found it to be overexpressed *in situ* in Kras arf$^{-/-}$GBMs compared to normal tissues (Figure 2*C*). In PDGFB oligodendrogliomas, HES1 expression was found to be comparable to normal tissue or, perhaps, to be slightly elevated on a cell-to-cell basis. These results suggest that, in Kras-induced GBMs, the Notch pathway is active because the receptor, ligand, and target are all expressed. In PDGFB-induced oligodendrogliomas, the receptor appears to be overexpressed; however, of the ligands and targets we examined, only moderate levels were detected. There may be some degree of Notch signaling through HES1 in oligodendrogliomas, but the level of activation is much less compared to that in Kras-induced tumors.

Notch Activation of Target Genes in Kras-Induced Tumors

Notch acts as a transcriptional activator by complexing with the DNA-binding protein CBF-1, displacing repressive factors and recruiting coactivators. Because one effect of Notch signaling is activation of transcription, an expression array analysis may reveal its function in Kras-induced tumors. We compared the expression profile of two Kras-induced tumors to the normal cortex from Ntv-a arf$^{-/-}$ mice (Table 1 and Table W1). The list of genes upregulated contained a wide range of proteins involved in various processes. Included in this list were some expected targets in Kras gliomagenesis. For example, glial progenitor markers, including vimentin, PDGFRα, and nestin, were increased. Ras signaling components and matrix-degrading enzymes were also increased. Of 828 genes upregulated by > 3-fold ($P < .05$), we searched for CBF-1 consensus sites (YGTGGGAA) in the 5-kb 5′ upstream promoter sequence in front of the transcriptional start site using the annotation provided on www.ensembl.org. Using this search, 136 genes were found to have this motif and to be potential Notch target genes by binding to CBF-1 (Table 1 and Table W1). Among this list of genes are ones that have been implicated by other studies as potential targets of Notch signaling, including cyclin D1 [35], S100a10 [36], and a Snail family member [37]. Not identified by this method but upregulated on the array and a potential Notch target is the cyclin-dependent kinase inhibitor p21 [38].

One interesting target found by this search is nestin, an intermediate filament protein that acts as a marker for neural progenitor cells. Although the 5′ upstream promoter plays a role in modulating its expression, it is the nestin second intron that directs expression in neural precursors [27,39]. Conserved near the 3′ end of the second intron of both human and mouse nestins is a CBF-1–binding site (Figure 4*A*). We tested whether Notch activation could activate transcription

Table 1. Selected List of Genes Upregulated By > 3-Fold in Kras $arf^{-/-}$ Tumors Compared to $arf^{-/-}$ Cortex.

GenBank Number	Gene Name	Protein	Fold 1	Fold 2
Structural				
NM_019390	*Lmna*	Lamin A	7.0	4.9
BG970109	*Lamb1-1*	Laminin B1 subunit 1	7.5	12.1
AV147875	*Vim*	Vimentin	12.1	16.0
AI413223	*Nes*	Nestin*	18.4	17.1
Matrix-interacting				
NM_008398	*Itga7*	Integrin α_7*	3.7	4.9
NM_008872	*Plat*	Plasminogen activator, tissue*	4.9	6.5
BM935811	*Itga6*	Integrin α_6	5.7	3.5
U37029	*Itgb1*	Integrin β_1 (fibronectin receptor β)	8.0	9.2
NM_013565	*Itga3*	Integrin α_3	9.8	6.5
NM_008873	*Plau*	Plasminogen activator, urokinase	18.4	29.9
NM_032007	*Mmp1b*	Matrix metalloproteinase 1b (interstitial collagenase)	64.0	42.2
BC019135	*Mmp12*	Matrix metalloproteinase 12	97.0	111.4
NM_053110	*Gpnmb*	Glycoprotein (transmembrane) nmb	119.4	59.7
Cell cycle				
BC027026	*Cdkn2c*	Cyclin-dependent kinase inhibitor 2C (p18)	4.0	6.1
X75483	*Ccna2*	Cyclin A2	4.9	4.6
AK007630	*Cdkn1a*	Cyclin-dependent kinase inhibitor 1A (P21)	6.5	5.7
NM_007631	*Ccnd1*	Cyclin D1*	9.2	9.8
AU015121	*Ccnb1*	Cyclin B1	26.0	27.9
Signaling				
NM_008008	*Fgf7*	Fibroblast growth factor 7	3.2	13.9
AW537708	*Pdgfra*	Platelet-derived growth factor receptor, α polypeptide	3.7	4.3
BC013066	*Dok1*	Docking protein 1	4.9	9.2
NM_008696	*Map4k4*	Mitogen-activated protein kinase kinase kinase kinase 4	5.3	4.3
BM947855	*Plk3*	Polo-like kinase 3 (*Drosophila*)	5.7	4.3
NM_010517	*Igfbp4*	Insulin-like growth factor–binding protein 4	5.7	4.0
BF681826	*Ralgps1*	Ral GEF with PH domain and SH3-binding motif 1	7.0	8.6
L07264	*Hbegf*	Heparin-binding EGF-like growth factor	7.5	12.1
NM_007484	*Rhoc*	Ras homolog gene family, member C	8.0	11.3
NM_007900	*Ect2*	ect2 oncogene	8.6	13.9
S69114	*Tgfbr2*	Transforming growth factor, β receptor II	9.2	19.7
NM_007568	*Btc*	β-Cellulin, epidermal growth factor family member	9.8	16.0
AV367068	*Dhh*	Desert hedgehog*	10.6	9.8
NM_133914	*Rasa4*	RAS p21 protein activator 4	26.0	11.3
NM_011950	*Mapk13*	Mitogen-activated protein kinase 13	45.3	36.8
Transcription and translation				
BM200591	*Eif1a*	Eukaryotic translation initiation factor 1A	3.0	3.0
BC013717	*Etf1*	Eukaryotic translation termination factor 1	3.2	3.7
BM120823	*Eif4e2*	Eukaryotic translation initiation factor 4E member 2	4.3	4.6
BC012674	*Ptrf*	Polymerase I and transcript release factor*	4.9	4.0
NM_133626	*Rrbp1*	Ribosome-binding protein 1*	9.8	7.0
Transcription factors				
AI746342	*Stat6*	Signal transducer and activator of transcription 6	3.7	4.3
BC006728	*Myc*	Myelocytomatosis oncogene	4.0	3.5
NM_011415	*Snai2*	Snail homolog 2 (*Drosophila*)*	4.6	26.0
BF017589	*Sin3b*	Transcriptional regulator, SIN3B (yeast)*	4.6	4.6
BC005686	*Elk3*	ELK3, member of ETS oncogene family	6.1	7.5
AB012278	*Cebpb*	CCAAT/enhancer binding protein (C/EBP), β	8.6	6.1
NM_007855	*Twist2*	Twist homolog 2 (*Drosophila*)	9.2	10.6
NM_009821	*Runx1*	Runt-related transcription factor 1	9.8	10.6
U34245	*Fosl1*	Fos-like antigen 1	11.3	6.1
BC003778	*Tcfap2c*	Transcription factor AP-2, γ	12.1	32.0
NM_033597	*Myb*	Myeloblastosis oncogene	14.9	6.5
BC019946	*Atf3*	Activating transcription factor 3	16.0	24.3
Other				
BB829652	*Nedd1*	Nedd1*	3.0	4.3
M12573	*Hspa1b*	Heat shock protein 1B	5.3	6.1
BC008152	*Casp1*	Caspase 1	6.5	13.9
AF220524	*Dnmt3l*	DNA (cytosine-5)-methyltransferase 3–like*	6.5	18.4
BC025083	*Glipr1*	GLI pathogenesis-related 1 (glioma)	8.0	13.0
NM_009112	*S100a10*	S100 calcium-binding protein A10 (calpactin)*	8.0	7.0
NM_009892	*Chi3l3*	Chitinase 3–like 3	362.0	157.6

Folds 1 and 2 represent fold changes in two tumors compared to the cortex.
*A gene that has a potential CBF-1–binding site in its 5-kb 5′ upstream promoter sequence.

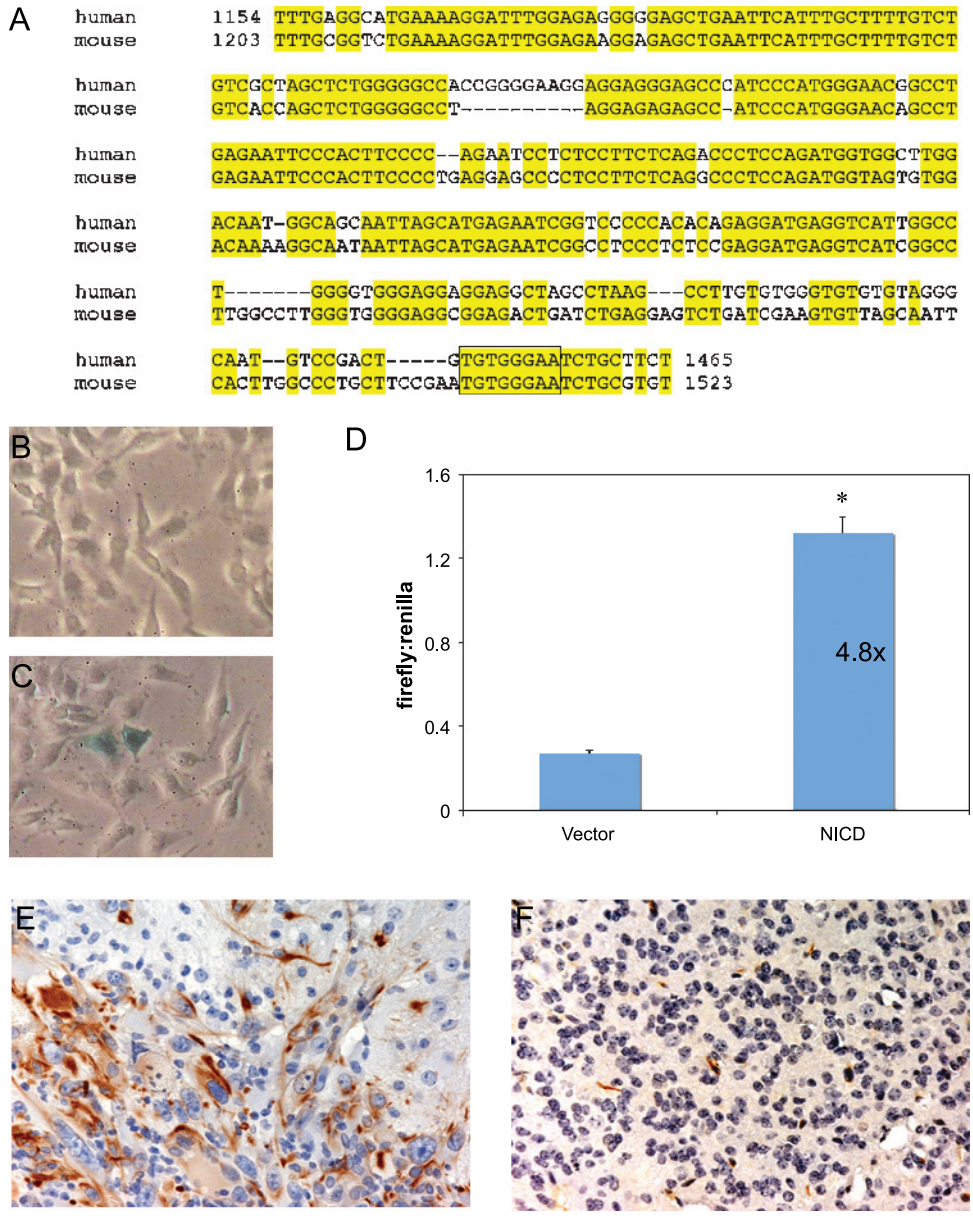

Figure 4. *Notch1 activation of the nestin second intron element. (A) Alignment of human and mouse* Nes *second intron segments. Boxed regions indicate potential CBF-1–binding site. Numbering based on GenBank sequences: human AF004335 and mouse AY438043. (B and C) Assay for β-galactosidase activity. U251 cells were transfected with a nestin–β-galactosidase reporter, along with either (B) empty vector or (C) NICD-expressing vector. (D) Luciferase assay of U251 cells transfected with nestin reporter along with either empty vector or NICD-expressing vector (*P < .01). (E) Nestin IHC in Kras-induced spindle tumor in Ntv-a arf$^{-/-}$ mouse. (F) Nestin IHC in PDGFB-induced oligodendroglioma.*

through this element. The human nestin second intron enhancer element linked to a tk minimal promoter is sufficient to replicate the neural expression pattern for nestin [28]. This enhancer–promoter combination was used to drive the expression of two reporter genes *β-galactosidase* and *luciferase*. Nestin reporters were tested in U251 glioma cell lines in the presence and in the absence of NICD expression. This form of Notch is equivalent to the proteolytically processed receptor, a constitutively active isoform [8]. We found that NICD could activate the promoter using either reporter. In the β-galactosidase assay, U251 cells cotransfected with NICD turned blue, whereas those cotransfected with empty vector did not (Figure 4, *B* and *C*). NICD also activated the luciferase reporter 4.8-fold compared to controls (Figure 4*D*). These data suggest that Notch may act directly to activate nestin expression in progenitor cells or glioma cells.

To assess whether activation of the Notch pathway correlated with nestin expression *in vivo*, we stained Ntv-a Kras arf$^{-/-}$ tumors and Ntv-a PDGFB tumors for nestin expression. In Kras-induced spindle-like tumors where the Notch pathway is active, nestin is expressed throughout the tumor (Figure 4*E*). However, in PDGFB-induced oligodendrogliomas where HES1, Delta1, and Jagged1 are not elevated to the same degree, nestin is not clearly expressed by tumor cells (Figure 4*F*). This shows that *in vivo* nestin expression correlates with activation of the Notch pathway.

Notch1 Activation Can Cooperate with Kras to Form Progenitor-Like Lesions

To establish the effect of Notch1 activation on glial tumors, we subcloned a myc-tagged mouse NICD isoform into the RCAS(A) retroviral system. The construct was tested for functionality by the ability to activate the Hes1 promoter (Figure 5*A*). To determine whether the expression of NICD in nestin-positive glial progenitors would be sufficient to induce tumorigenesis, we infected 39 Ntv-a mice with either RCAS NICD virus or RCAS NICD virus with an empty RCAS virus on postnatal day 1 and observed them for symptoms. We analyzed 21 mice up to 12 weeks and 18 mice up to 24 weeks. None of these developed any signs of tumor, and histologic analysis of the brains yielded normal-appearing results (Figure 5*B*). To determine whether Notch signaling could cooperate with other oncogenes, we coinfected NICD

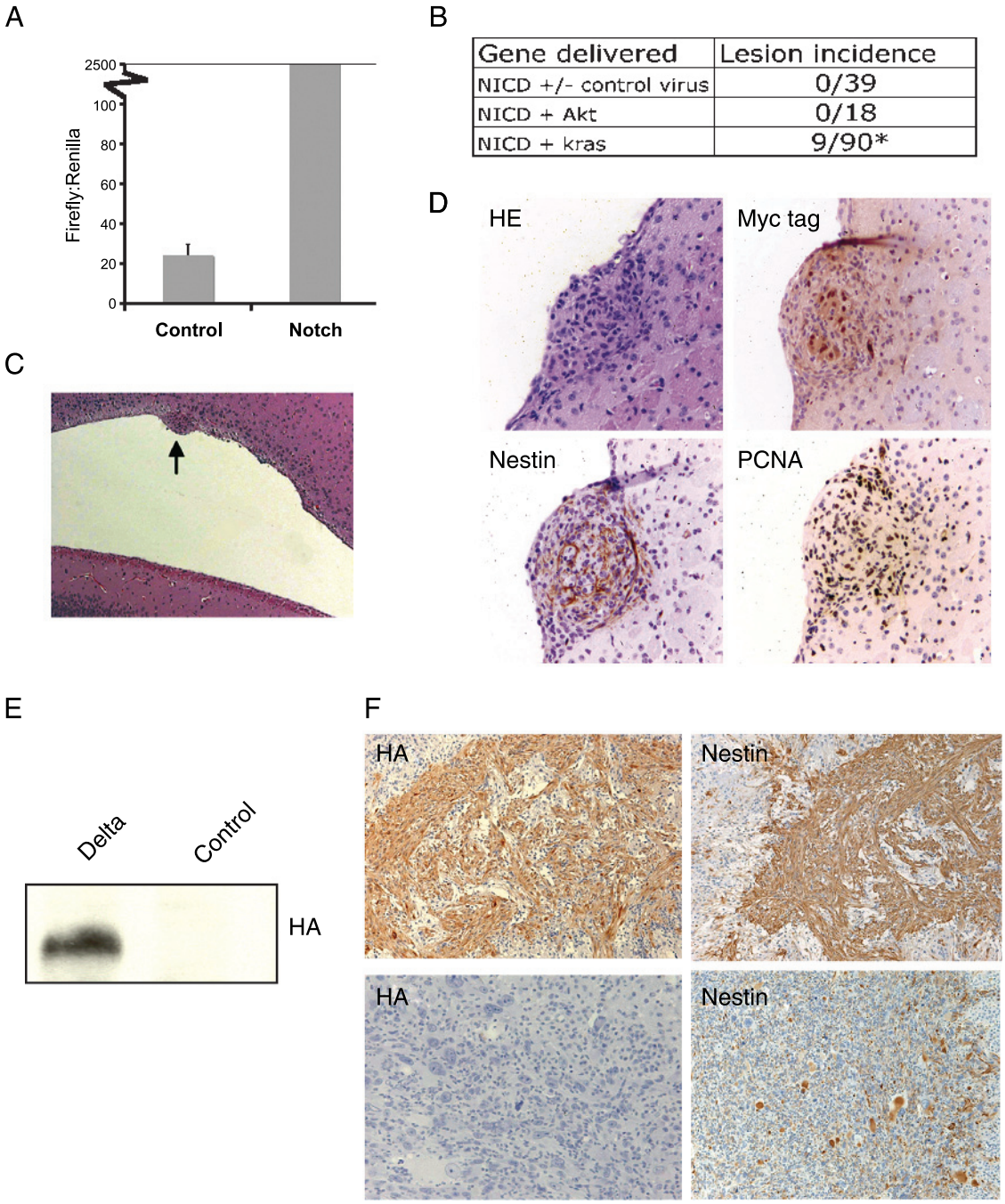

Figure 5. *Activated Notch and Kras can induce lesions in Ntv-a–targeted mice. (A) HES1 promoter luciferase assay with empty plasmid control or RCAS NICD transfection. (B) Table of lesion incidence in NICD/NICD + control virus, NICD + Akt, and NICD + Kras infections of Ntv-a mice (*P < .05). NICD + Kras compared to NICD/NICD + control virus. (C) H&E of NICD + Kras lesion located in the SVZ. (D) Kras + NICD lesion H&E, myc tag IHC of myc-tagged NICD, nestin IHC, and PCNA IHC. (E) HA Western blot analysis of cell lysates from rDelta1-HA–transfected and control-transfected cells. (F) IHC of HA (rDelta1) and nestin expression from a Kras + rDelta1-HA–generated tumor in Gtv-a arf$^{-/-}$ mice.*

virus with a virus carrying either activated Akt or Kras—oncogenes previously identified to contribute to glial tumorigenesis [25]. Furthermore, the delivery of these oncogenes alone in a wild-type background is insufficient to generate tumors or lesions in this mouse model [25]. The combination of NICD and Akt failed to generate any distinguishing abnormalities in 25 tested mice analyzed up to 24 weeks. However, the combination of NICD and Kras produced periventricular lesions in 9 of 90 infected mice. Twenty-nine mice were analyzed up to 12 weeks, and 61 mice were analyzed up to 24 weeks. Regardless of the age of the mice in which they were found, lesions remained small and limited to the SVZ without progressing to tumors or larger lesions (Figure 5*C*). This incidence of lesion formation was statistically significant ($P < .05$) when compared to mice infected with RCAS NICD virus alone or combined with a control RCAS virus. These lesions were then analyzed for immunohistochemical markers (Figure 5*D*). They are positive for the myc tag on the NICD, indicating effective viral delivery, and are also positive for nestin and PCNA, indicating that cells were in active cell cycles. This result confirms *in vivo* the potential for nestin to be a direct Notch transcriptional target. This also suggests that Notch activation can cooperate with Kras to produce lesions that retain stem-like character by their location in the SVZ, continued proliferation, and progenitor marker expression. Nestin expression is maintained either through Notch's direct action on its promoter or through Notch's effect on maintaining an undifferentiated state in nestin-positive progenitors.

Ntv-a mice support gene transfer to nestin-expressing cells; in this case, Notch activity may simply maintain nestin expression. We next used Gtv-a mice to determine the ability of Notch activation to drive nestin expression in tumors derived from a GFAP-expressing cell of origin, a more differentiated cell type that has already lost nestin expression. We cloned an HA-tagged version of rat Delta1 (a ligand for Notch receptor) into the RCAS vector and assessed its expression using Western blot detection of the HA tag in Ntv-a mouse–derived glial cells (Figure 5*E*). We then infected Gtv-a $arf^{-/-}$ mice with RCAS-Kras, RCAS-rDelta1-HA, or a combination of the two. Gtv-a $arf^{-/-}$ mice express the viral-targeting receptor on GFAP-positive cells, and RCAS-mediated Kras oncogene delivery to these cells induces a similar spindle tumor with moderate nestin expression, as seen with Ntv-a $arf^{-/-}$ mice [29]. These tumors have abundant Notch1 expression, as determined by *in situ* analysis. Expression of rDelta1-HA alone in these mice was insufficient to induce tumors. Mice infected with a combination of RCAS-Kras and RCAS-rDelta1-HA developed tumors and were sacrificed when symptomatic or at 12 weeks. These tumors were then analyzed for HA and nestin expression. HA-staining regions are composed of Kras-driven tumors that have additional Notch activation by the expression of rDelta1. In analyzing the five large tumors that developed, in regions where Kras alone was expressed (with no HA staining), nestin expression was intermediate throughout (Figure 5*F*). However, nestin expression was markedly elevated where rDelta1 was coexpressed (as determined with positive HA staining) with Kras (Figure 5*F*). This demonstrates that elevated Notch signaling upregulates nestin expression in glial tumors, even from a GFAP-expressing cell of origin, and can collaborate with Kras signaling to alter tumor phenotype.

Discussion

Our study demonstrates that Notch signaling can play important roles in glial tumor development, particularly in promoting nestin expression that may contribute to stem cell potential. We were able to identify Notch receptors and ligands in human glial tumors, suggesting that juxtacrine or autocrine modes of activation are possible—a situation that most often occurs in GBMs, the highest grade of glial tumors. Previous results have indicated higher Notch1 mRNA in grade II to grade III astrocytomas than in GBMs, no significant elevation of Delta-like1 mRNA in astrocytomas and GBMs, and elevation of Jagged1 in only a subset of GBMs [23]. Our results agree with this previous report on the elevated level of Jagged1 in a subset of GBMs. However, at the protein level, we observed similar levels of Notch1 receptor in human glial tumors and higher ligand levels in GBMs. The lack of correlation between mRNA and protein levels is consistent with previous reports showing that Notch1 is one mRNA that is translationally regulated through its recruitment into polyribosomes by signaling pathways known to be active in GBMs [6]. In this study, we observed a similar finding in mouse tumors with elevated Notch1 protein in Kras-induced GBMs, where Ras and Akt pathways are significantly elevated. Thus, equivalent mRNA levels may lead to differing amounts of protein. We have also identified Notch2 protein to be expressed in selected GBMs. Thus, our results add to the growing literature supporting the importance of Notch signaling in the formation of human GBMs.

In our mouse glial tumor models, we found that, in Kras-induced GBMs, Notch1 receptor, Delta-like1, and Jagged1 are upregulated, accompanied by increased transcription of HES1, a common Notch transcriptional target. The cooperative nature of activated Notch and Kras signaling was additionally observed with the ability of these two genes combined to generate lesions located in the SVZ, the location of neural stem cells. The cells in these lesions continue to express a proliferation marker and also nestin, much as stem-like cells do. The ability of Notch to sustain the expression of the progenitor marker nestin may have a direct effect on transcription by Notch at the nestin second intron enhancer element. Based on these findings, Notch activation appears capable of promoting or sustaining nestin expression and the stem-like character of SVZ cells.

Given the diverse roles that the Notch pathway plays in normal glial development, parallels between development and tumor formation may be seen. As in development, persistent Notch activation may be a means to keep cells in a more undifferentiated progenitor state. It is believed that cancer stem cells may be the source of tumor cells and that SVZ is thought to be the origin of neural stem cells in the cerebrum [40]. The periventricular lesions seen with combined Notch and Kras infections in wild-type mice may be a

reflection of this aspect of Notch's function. In normal development, nestin-positive cells migrate from the SVZ into the brain on postnatal day 0 and are limited to a small zone of periventricular cells in the adult brain [41]. By activating the Notch and Ras pathways in nestin-positive progenitors, these cells appear capable of continued proliferation in adult mice in the SVZ as if they have been prevented from proceeding through a normal differentiation pathway.

In Kras-generated GBMs, the activation of the MAP kinase Erk and Akt pathways may affect protein translation. Thus, compared to PDGFB-generated oligodendrogliomas, Kras-induced GBMs produce more Notch proteins from relatively similar amounts of Notch mRNA. The expression of Notch ligands Delta1 and Jagged1 appears to be regulated by transcriptional mechanisms that upregulate expression in Kras tumors and not in PDGFB tumors. The coexpression of ligand and receptor in Kras-induced GBMs may be responsible for juxtacrine Notch signaling that can maintain progenitor characteristics. Therefore, nestin expression is correlated with Kras-induced GBM-like tumors and not with PDGFB-induced oligodendrogliomas. In PDGFB-generated oligodendrogliomas, the Notch1 receptor is overexpressed, but the ligands and Hes1 target are either minimally expressed or at levels slightly higher than that in normal brain. This, however, does not exclude Notch function from these tumors. There are three Delta-like isoforms and two Jagged isoforms, in addition to a newly identified ligand F-contactin that has been shown to have specific effects on oligodendrocytes [42]. Alternatively, the lower level of receptor and ligand expression seen in Western blot analysis may still be sufficient for signaling.

Inhibition of Notch activity may be useful for glioma therapy. One possible target is presenilin γ secretase, which acts to cleave and release Notch from membranes. The combination of a Notch inhibitor and Ras inhibitor may be particularly effective because these pathways seem to work synergistically to induce lesions and to sustain Ras effects. Further studies that elucidate the molecular mechanisms and specific targets of Notch signaling, particularly ones that distinguish its multiple functions, will also bring greater insight into glioma biology.

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Table W1. Genes with Potential CBF-1–Binding Sites in the 5-kb 5′ Upstream Promoter Sequence from the Transcriptional Start Site (as Defined By ensembl.org).

Genes with Potential CBF-1–Binding Sites				
Adamts1	*Cma2*	*Hist1h4i*	*Ncf4*	*S100a4*
Ahnak	*Csf2rb1*	*Hist2h2aa1*	*Nedd1*	*Saa3*
Akap12	*Ctsz*	*Hist2h3c1*	*Nes*	*Samhd1*
Angptl4	*Cxcl4*	*Hist3h2a*	*Niban*	*Sdfr2*
Anxa1	*Dhh*	*Hmga1*	*Nid2*	*Serpine1*
Anxa4	*Dnase1l1*	*Ibsp*	*Nmt2*	*Sgol1*
Ap2b1	*Dnmt3l*	*Icsbp1*	*Nusap1*	*Sin3b*
Aqp1	*Emilin1*	*Igsf6*	*Ostf1*	*Slc16a3*
Baiap2l1	*Emp1*	*Il1r2*	*P4hb*	*Slc20a2*
BC027061	*Ezh2*	*Itga7*	*Pcolce*	*Slc25a24*
Bfsp1	*F7*	*Itpr3*	*Pcolce2*	*Slfn1*
C1qb	*Fabp4*	*Kdelr2*	*Plat*	*Snai2*
C1qtnf1	*Fblim1*	*Kdelr3*	*Plaur*	*Spata6*
Calca	*Gbp2*	*Kif4*	*Plp2*	*Spp1*
Cald1	*Gem*	*Lama4*	*Podxl*	*Stk17b*
Car13	*Glipr2*	*Lcp1*	*Ppap2c*	*Stk3*
Cask	*Gpr35*	*Lgals3*	*Prlpb*	*Tagln2*
Ccl24	*Guca1a*	*Lgals7*	*Ptpn6*	*Tax1bp3*
Ccnd1	*H2-Aa*	*Lmnb1*	*Ptrf*	*Tcfec*
Cd244	*H2-Ab1*	*Loxl1*	*Ptx3*	*Tfpi2*
Cd274	*Hdlbp*	*Ltb4dh*	*Pxn*	*Thbs1*
Cd300lf	*Hist1h2ad*	*Mcpt1*	*Rgs18*	*Timp1*
Cd9	*Hist1h4f*	*Myh9*	*Rrbp1*	*Tnfaip2*
Cflar	*Hist1h4h*	*Myo1c*	*S100a10*	*Ugt1a2*