**Notch3 Activation Promotes Invasive Glioma Formation in a Tissue Site-Specific Manner**

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**Abstract**

Although Notch signaling has been widely implicated in neoplastic growth, direct evidence for *in vivo* initiation of neoplasia by the pathway in murine models has been limited to tumors of lymphoid, breast, and choroid plexus cells. To examine tumorigenic potential in the eye and brain, we injected retroviruses encoding activated forms of Notch1, Notch2, or Notch3 into embryonic mice. Interestingly, the majority of animals infected with active Notch3 developed proliferative lesions comprised of pigmented ocular choroid cells, retinal and optic nerve glia, and lens epithelium. Notch3-induced lesions in the choroid, retina, and optic nerve were capable of invading adjacent tissues, suggesting that they were malignant tumors. Although Notch3 activation induced choroidal tumors in up to 67% of eyes, Notch1 or Notch2 activation never resulted in such tumors. Active forms of Notch1 and Notch2 did generate a few small proliferative glial nodules in the retina and optic nerve, whereas Notch3 was 10-fold more efficient at generating growths, many of which were large invasive gliomas. Expression of active Notch1/Notch3 chimeric receptors implicated the RBPjk-association molecule and transactivation domains of Notch3 in generating choroidal and glial tumors, respectively. In contrast to our findings in the optic nerve and retina, introduction of active Notch receptors, including Notch3, into the brain never caused glial tumors. Our results highlight the differential ability of Notch receptor paralogs to initiate malignant tumor formation, and suggest that glial precursors of the optic nerve, but not the brain, are susceptible to transformation by Notch3. *Cancer Res;* 71(3); 1–12. ©2011 AACR.

**Introduction**

Notch signaling plays a critical role in the specification, proliferation, and survival of stem/progenitor cells in a number of tissues, including the central and peripheral nervous systems (1). The pathway is also widely implicated in neoplasia, and in most contexts promotes neoplastic growth (2), but can act as a tumor suppressor in some cell types (3–5). Notch is activated in a broad range of hematopoietic and solid tumors (2–4, 6–12). Its role in leukemogenesis is probably best described, as activation of the pathway can promote murine T-ALL, similar to that seen in humans (8, 11, 13, 14).

Notch is thought to play a particularly important role in poorly differentiated tumor cells, where pathway inhibition may be able to deplete "cancer stem cells," which are resistant to radiation and standard chemotherapies (1, 5, 7, 9, 10, 12, 15–18). Small molecules targeting Notch have shown great promise in preclinical testing of several tumor models. On the basis of such studies, phase I clinical trials for leukemia and breast cancer have been initiated using gamma-secretase inhibitors that block the activation of Notch receptors (Clinical Trials.Gov identifiers NCT00106145; NCT00878189). However, for such therapies to be optimally utilized, we must first more fully understand the complexities of Notch signaling in cancer, as its effects are context-dependent (5, 16).

The role(s) of the various Notch receptors in tumor initiation *in vivo* are particularly poorly understood. Though structurally similar, the 4 mammalian Notch paralogs (Notch1–4) are not functionally equivalent in all contexts. The mechanistic basis of such differences is still being uncovered, but it has been shown that the various Notch receptors can relate uniquely to binding site distribution and orientation on target gene promoters (19).

In this study, we examined potential functional distinctions between the Notch paralogs by introducing activated Notch receptors into the brain and eye, and we identified proliferative lesions in the retina, optic nerve, and lens. Importantly, we...
found that active forms of Notch1, Notch2, and Notch3 have differing abilities to induce tumors, and that glial progenitors in the optic nerve and retina are particularly susceptible to Notch3-driven transformation as compared with those in the rest of the central nervous system. These results highlight the sometimes distinct capacities of various Notch receptors to induce tumor formation and the context-dependence of glial progenitor transformation.

Materials and Methods

NICD1/NICD3 chimeric constructs
The NICD1/NICD3 chimeric human proteins were expressed using the retroviral vector pCLE (20).The chimeric cDNAs were made using standard PCR and ligation protocols, and were confirmed by DNA sequencing. Briefly, we amplified 3 different segments of Notch1 intracellular domain (NICD1) and 3 of NICD3, each product varying in length to include different regions. For NICD1 these were called ΔN1xxx, ΔN11xx, and ΔN111x, and included the RBPsj-association molecule (RAM) domain and adjacent downstream residues, RAM, RAM/Ank1–2, and RAM/Ank1–5, respectively (Ank, ankyrin repeat). For NICD3, these were called ΔNxx33, ΔNxx33, which included TADA (a proximal portion of the transactivation domain), Ank3–5/TADA, Ank1–5/TADA, respectively. We already possessed the human ΔN1111 and ΔN1333 constructs in the retroviral vector pCLE (ΔN1CLE and ΔN3CLE; ref. 21). All primers were designed to include exogenous restriction enzyme sites, or sites created by silent point mutations, to permit generation of chimeric constructs by ligation (e.g., ΔN11xxx was ligated to ΔNxx33). ΔN1111 encodes residues 1761–2093 of Notch1 and 2006–2098 of Notch3. ΔN1133 encodes residues 1761–1993 of Notch1 and 1905–2098 of Notch3. ΔN1333 encodes residues 1761–1858 of Notch1 and 1767–2098 of Notch3. See Supplementary Figure S1 for the amino acid sequence of the chimeric proteins.

Constructs and virus preparation
Retroviral constructs expressing the activated forms of Notch1–3 (human Notch intracellular domain (NICD1, NICD2, NICD3) or the NICD1/NICD3 chimeras and adjacent alkaline phosphatase (placental alkaline phosphatase; PLAP) reporter were generated using standard protocols (20). The NIC1CLE (22), ΔN2CLE, ΔN3CLE, ΔN1113CLE, ΔN1133, and ΔN1333CLE-expressing viruses encode truncated NICD that lacks a portion of the C terminus, a modification required to obtain high viral titer. ΔN1CLE encodes residues 1761–1916; ΔN2CLE encodes residues 1703–2146; and ΔN3CLE encodes residues 1663–2098. The N3CLE-expressing virus encodes full-length NICD3, residues 1663–2321 (21). Virus expressing empty vector (CLE) was used as a control.

Animals and in utero viral injections
The CD1 (Charles River) and Black Swiss (Taconic) mice used in this study were maintained in accordance with the Institutional Animal Care and Use Committee at Johns Hopkins University School of Medicine. Embryos were injected in utero at E9.5 or E10.5 with virus using ultrasound guidance as described previously (20). All viruses used for injection had titers of $1 \times 10^5$ to $4 \times 10^8$. The animals were sacrificed at 30 to 60 days of age, and occular and brain tissues were harvested.

Staining of tissue sections and analysis
Eye and brain tissue obtained from virally injected mice was fixed in 4% paraformaldehyde for frozen sections or formalin for paraffin-embedded sections. Sectioning, PLAP staining (22), hematoxylin and eosin (H&E) staining, and immunohistochemistry were carried out using standard methods. We blindly scored multiple H&E stained adult eye sections from virally injected animals. Immunohistochemistry on paraffin sections was carried out using the Vectastain ABC kit and DAB Peroxidase Substrate kit (Vector Laboratories) as specified by the manufacturer's protocol. Primary antibodies used were α-Notch3 (rabbit, 1:1,000; Santa Cruz Biotechnology), α-Pax6 (mouse, 1:400, Millipore Corporation), α-Chx10 (sheep, 1:100, Exalpha Biologicals), α-GFAP (rabbit, 1:1,000; DAKO), α-Nestin (chicken, 1:250; Aves Labs, Inc.), and Hes5 (rabbit, Gaiano Laboratory, only recognizes overexpression). Alexa Fluor Dye conjugated secondary antibodies used were obtained from Invitrogen.

Luciferase assay
Luciferase assays were conducted on E14.5 mouse cortical neural progenitors or NIH3T3 cells transfected with the Hes5promoter (Hes5p)- or CBF1RE [4xCBF1 (C promoter-binding factor 1)-responsive element]-luciferase constructs along with one of the Notch-expressing constructs, according to the manufacturer's protocol (Lonza). The Hes5 promoter was a gift from Toshiyuki Ohnatsuka at Kyoto University and the CBF1RE-luciferase construct was a gift from Diane Hayward at Johns Hopkins University. Cells were harvested 48 hours after transfection and luciferase activity was measured (Promega). Each sample was normalized to beta-galactosidase expression (Clontech).

Preparation of rat astrocyte cell cultures
The cortex and optic nerves were isolated from postnatal day 2 Sprague-Dawley rats as described previously (23, 24). The optic nerve and retinal cultures were 90% glial fibrillary acidic protein (GFAP)-immunopositive, and the cortical cultures were 60% GFAP-immunopositive (data not shown). Murine ΔN1CLE (22) caused the same incidence of ocular tumors as ΔN1CLE and was used to infect these cultures.

Quantitative PCR
RNA was obtained from NIH3T3 cells or rat optic nerve and cortical astrocyte cultures infected with the Notch-expressing viruses and real-time polymerase chain reaction (RT-PCR) was conducted using SYBR Green reagent (Applied Biosystems) with all samples run in triplicate on an iCycler (Bio-Rad). Values were normalized to actin or GAPDH using the standard curve method. The following primer sequences were used. Mouse Hes1 Forward (F): 5'-AACGACATCGTCCCAGGTTTTG-3'; mouse Hes1 Reverse (R): 5'-AAAGCCTATCATGGAGAAGAGGCG and ΔN1CLE and was used to infect these cultures.

OF2 Cancer Res; 71(3) February 1, 2011

Published OnlineFirst January 18, 2011; DOI: 10.1158/0008-5472.CAN-10-0690
In contrast, injection of control CLE virus was never associated with gross or microscopic lens abnormalities. This was an ongoing proliferative process, as evidenced by the presence of mitotic figures and Ki67-positive cells (Fig. 1Dii inset and iii), with 11% of cells expressing this proliferative marker in the depicted cataract. The thickened epithelial layers stained positive for PLAP (Fig. 1Diii), indicating that viral infection was directly associated with the hyperproliferative lesions. Another group recently reported that active Notch1 signaling also induced ALE hyperproliferation (32). Our N3CLE-induced lesions seem very similar to human anterior subcapsular cataracts (ASC), which are also characterized by a multilayered lens epithelium and abnormal deposition of capsular material (33).

Pigmented choroidal tumors are generated by Notch3 signaling

N3CLE-injected animals also developed invasive tumors arising in the choroidal layer of the eye, a loose fibrovascular tissue containing scattered melanocytes. The invasive choroidal neoplasms, comprised of oval and spindled cells, were present in 63 of the 94 eyes (67%) examined in N3CLE-injected animals (Table 1). Such tumors were never observed in control CLE-injected eyes (n = 22). Tumors were identified in all regions of the choroid, from the periphery (Fig. 2A, arrow) to the posterior pole, and some eyes contained multiple separate lesions in various regions. The sheets and clusters of tumor cells were highly proliferative, as evidenced by the presence of numerous mitotic figures and Ki67-positive cells (Fig. 2Biii). PLAP staining (Fig. 2A and Biii) and nuclear Notch3 immunopositivity (Fig. 2Bii) indicated that the choroidal lesions arose from cells from N3CLE infection with increased Notch activity. Many of the larger choroidal tumors invaded through transscleral canals into the orbital space (Fig. 2Bii). A few tumors extensively infiltrated the periocular tissues and were grossly apparent on external examination as exophytic lesions around the eye. The fact that not all eyes injected with N3CLE developed these neoplasms suggests that additional genetic alterations may be needed to promote tumorigenesis, although limited sampling may have also affected the number of lesions detected.

The precise pathological classification of these neoplasms is not clear. They did not seem embryonal, and were not immunopositive for Nestin, synaptophysin, TuJ1, GS, PAX6, or GFAP, suggesting that they were not neuronal or glial. In humans, uveal melanoma is the most common malignant neoplasm of the choroid. A subset of injections was performed into the pigmented Swiss Black mouse line, and choroidal tumors in these animals always contained pigmented cells (Fig. 2Bi). The tumors clearly arose from choroid rather than retinal pigment epithelium, as the latter pigmented cell layer was intact in all of the smaller lesions examined (Fig. 2Bii, arrow and data not shown). Choroidal melanocytes derive from neural crest, a tissue that would be at least partially associated with gross or microscopic lens abnormalities. This was an ongoing proliferative process. The thickened epithelial layers stained positive for PLAP (Fig. 1Diii), indicating that viral infection was directly associated with the hyperproliferative lesions. Another group recently reported that active Notch1 signaling also induced ALE hyperproliferation (32). Our N3CLE-induced lesions seem very similar to human anterior subcapsular cataracts (ASC), which are also characterized by a multilayered lens epithelium and abnormal deposition of capsular material (33).

Constitutive Notch3 signaling generates retinal lesions that express retinal progenitor markers

At E9.5 to E10.5 the developing forebrain and optic cup form a continuous open structure, allowing viral infection of anlage giving rise to the retina (Fig. 1A). PLAP staining of the optic cup neuroepithelium in animals sacrificed 6 days post viral injection confirmed infection of these cells (Fig. 1Bi, arrow). Animals injected with N3CLE retrovirus encoding constitutively active Notch3 developed retinal lesions in 62 of 94 adult eyes (66%) examined (Table 1). N3CLE-induced retinal lesions were focal and varied in size. Some disrupted only the inner retinal layer (Fig. 1Bii) whereas others affected all 3 retinal layers (Fig. 1Biii). The retinal lesions coincided with PLAP staining and nuclear Notch3 immunoreactivity (Fig. 1Biv and v) indicating that they were induced by Notch3 activity.

To identify the cell types contained within the N3CLE-induced retinal lesions, we used immunofluorescence to detect various markers of retinal differentiation. The disorganized regions contained some cells expressing the glial marker glutamine synthetase (GS) and the early neuronal marker BiIII-tubulin (Fig. 1Biv and v). Interestingly, within the lesions we also identified numerous cells-expressing Pax6 or Chx10 (Fig. 1C), and a subpopulation coexpressing both of these markers (Fig. 1C, arrowheads). Occasional cells were Ki67-positive, indicating a degree of proliferation. However, the lesions did not have microscopic features of a retinocytoma or a retinoblastoma. Previous studies have shown that retinal stem/progenitor cells coexpress Pax6 and Chx10 during early eye development; their expression later segregates and becomes cell specific (25–27). Pax6/Chx10 coexpression within the retinal lesions therefore suggests the presence of stem/progenitor-like cells in which Notch pathway deregulation has inhibited normal differentiation. Prior reports have shown that Notch1 activity promotes progenitor cell character in the developing retina (28–30), and our findings suggest that Notch3 can play a similar role.

Constitutive Notch3 signaling causes overproliferation of lens epithelium

Many mice injected at E9.5 or E10.5 with N3CLE developed white, cataractous eyes by 1 month of age (Fig. 1C). Lens formation is initiated early in fetal development and at E10.5 the ectothermal lens placode is still located on the surface of the embryo (31). In the normal mature lens, the only remaining cells are an anterior lens epithelium (ALE) monolayer below the capsule. On microscopic examination, we observed that 22 of 94 eyes (23%) from animals injected with N3CLE showed overproliferation of lens epithelium with adjacent deposition of capsular material (Fig. 1Dii; Table 1). In some, almost all of the epithelium was multilayered, whereas in others normal-looking epithelium (Fig. 1Dii, arrowhead) was present next to regions 10 or more cells thick (Fig. 1Dii, arrow).
Figure 1. Retinal lesions and ASCs are caused by Notch3 signaling. A, virus is microinjected into the ventricles of an E10.5 mouse embryo. At the time of injection, the forebrain and optic cup are a continuous structure, allowing viral infection of the anlage giving rise to the retina and optic nerve. Viral particles are released into the amniotic sac near the injection site on needle withdrawal and infect the developing lens. B, E17.5 retina in a N3CLE-injected animal with a PLAP-positive clone (i). H&E staining showed that N3CLE-induced retinal lesions can span 1 (ii) or all 3 layers (iii) of the retina. Retinal lesions contained BIII-tubulin-positive (iv) and GS-positive (v) cells that were often double positive for nuclear Notch3 (N3). C, Pax6/Chx10 double-positive cells in retinal lesions (arrowheads). D, i, white cataractous lenses developed in N3CLE-injected animals. ii, the ALE (arrowhead) expanded into multiple epithelial layers (arrow) and contained mitotic bodies in the N3CLE-induced ASCs (inset). iii, areas of hyperproliferation were PLAP positive and contained Ki67-positive cells (DAPI counterstain). Original magnifications: ×20 (Bi, Biv, and C), ×40 (Bv and Di), ×200 (Bii, Biii, and Di).
melanomas at this site. However, we were unable to verify melanocytic differentiation in the lesions using several immunohistochemical markers (data not shown). The multilayered concentric growth of the tumors in focal regions also shows some similarity to myopericytoma, although in humans such tumors are benign and are not known to occur in the eye. Thus, the classification, grade, and relationship of these choroidal tumors to a specific type of human neoplasm is unclear at this point.

Notch3 signaling induces glial tumors in the retina and optic nerve

Histological examination revealed that 23 of 94 eyes (24%) from the N3CLE-injected animals developed GFAP-positive glial lesions in the retina (Table 1; Fig. 2C and D). Some of these were relatively small (Fig. 2C), but most extended along large portions of the retina, and these often invaded through the retinal pigment epithelium and into the choroid (Fig. 2Di). In 2 cases, retinal glial tumors invaded through scleral canals and into the orbit. Because of this highly invasive behavior and the presence of scattered mitotic figures, we believe these are glial neoplasms rather than reactive gliotic lesions.

Glial tumors of similar appearance were also identified in the optic nerves of 20 of 94 N3CLE-injected animals (21%; Table 1). These tumors were only moderately cellular, with abundant cytoplasm and some elongated cytoplasmic processes (Fig. 3A). Occasional mitotic figures and Ki67-positive cells were present (Fig. 3Aii inset and Ci). In some tumors, neoplastic cells were localized under the meninges and focally distended the coverings of the optic nerve (Fig. 3A, arrowheads); in others, the optic nerve was more diffusely infiltrated by tumor. In 16 of the 20 eyes with optic nerve tumors (80%), the neoplasm invaded into the soft tissues surrounding the nerve. This most commonly occurred in the region of the optic disk, but in some optic nerves greatly distended by tumor, strands of neoplastic cells exited through more posterior channels penetrating the nerve sheath (Fig. 3Bi). Other glial lesions involved both the optic nerve and retina. In total, we

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NOTE: Virally infected eyes were blindly scored. The percent occurrence was rounded to the nearest whole number.

Figure 2. Invasive choroidal tumors and retinal glial lesions arise following Notch3 activation. N3CLE-induced cellular choroidal tumors in the adult eye were PLAP positive (A), pigmented, and arose in the choroidal layer beneath an intact retinal pigment epithelium (Bi, arrow). Many choroidal tumors invaded outward through breaks in the sclera (Bi, arrowheads) and could diffusely infiltrate periciliary tissue. Choroidal tumors contained nuclear N3 and Ki67-positive cells (Bi and Dii), C and D. N3CLE-injected animals contained PLAP-positive retinal glial lesions (C), GFAP-positive (Dii), and GS-positive (Diii). Original magnifications: ×10 (A), ×20 (Bi), ×40 (Bi and Dii), ×63 (Bii, Dii, and Diii), ×64 (Bi), ×200 (C).
identified invasive glial tumors in the orbital tissues of 47 of 94 eyes injected with N3CLE (50%; Table 1). Although many of these were clearly associated with retinal or optic nerve gliomas, others were not contiguous with these neural structures in the sections examined. We believe that most or all of these were associated with optic nerve or retinal gliomas that were not identified in limited tissue examined microscopically, but some could be distinct lesions arising from orbital neural tissues.

As was true for the choroidal tumors induced by NICD3, both PLAP and nuclear Notch3 (Fig. 3C1 and D) were identified in all optic nerve and retinal tumors examined, and no glial lesions were identified in animals injected with control CLE virus. We believe these tumors are astrocytic because they strongly express GFAP when examined using immunohistochemistry (Figs. 2Dii, 3Bi and D), and frequently contained elongated cellular processes similar to those in human astrocytoma. Nestin, an intermediate filament often expressed in neural progenitor cells and glial tumors, was diffusely present in the proliferative lesions (Figs. 3Cii and D). The Nestin- and GFAP-positive cells within the glial lesions also coexpressed nuclear Notch3 and the downstream target Hes5 (Fig. 3Cii and D), and PLAP (not shown) on immunofluorescent analyses. The choroidal tumors, which were sometimes encountered in the same eyes as invasive gliomas, were negative for GFAP and Nestin (data not shown).

**Notch1 and Notch2 activation does not efficiently generate ocular tumors**

The mammalian genome contains 4 Notch receptors, 3 of which (Notch1–3) are very similar in sequence, although Notch1 and Notch2 are more similar to each other than to Notch3 (35, 36). Despite their high level of identity, the effects of the various Notch receptors are not always the same, and in some contexts they can even have opposing effects (4). We therefore sought to determine whether introduction of either activated Notch1 or Notch2 would produce proliferative ocular lesions similar to those described above.

To generate high-titer virus expressing constitutively active Notch1 and Notch2, we found that we needed to truncate a portion of the NICD C terminus. Because N3CLE expresses full-length NICD3, we also generated a shortened NICD3-expressing virus (ΔNICD3) that contained a comparable C-terminal truncation (Fig. 4A). The resulting viruses all infected
the retina and optic nerve. The overall extent of infection as measured by PLAP staining was equivalent for DN1CLE, DN3CLE, and N3CLE (Fig. 4B). Some animals infected with DN2CLE also showed comparable levels of PLAP in these regions (Fig. 4B), but a significant percentage had less extensive staining despite injection with the same viral titers.

All constructs were capable of inducing the CBF1-dependent Notch targets Hes1 and Hey1 to a similar level as N3CLE in NIH3T3 cells (Fig. 4C). RT-PCR showed that the Notch-expressing constructs increased Hes1 and Hey1 expression to similar levels in NIH3T3 cells when compared with CLE. Notch-expressing constructs increased reporter expression from the Hes5 promoter (Hes5p-luciferase) construct in E14.5 cortical neural progenitors. We have also previously shown similar effects for the activated Notch1 and Notch3 constructs in neural progenitors in the developing brain (21, 22). Importantly, DN3CLE-injected animals developed retinal lesions, cataracts, and glial tumors with a similar incidence as those observed in the N3CLE-injected animals (Table 1), suggesting that the truncation does not affect overall Notch activity or tumor initiation capacity in vivo.

Retinal disorganizations with an appearance and immunophenotype identical to those induced by activated Notch3 were present in 20% of eyes injected with DN1CLE, indicating that the construct was biologically active in vivo. Both DN1CLE and DN2CLE could also induce proliferative cataracts in the lens, but in contrast to N3CLE and DN3CLE they were not able to generate large tumors in the eye. The most pronounced differences following Notch activation were in the choroid, as animals injected with DN1CLE and DN2CLE never developed tumors in this tissue. Glial lesions were also decidedly less common in the DN1CLE-injected animals. Only very small, noninvasive retinal, and optic nerve glial proliferations were observed.

**Figure 4.** The Notch-expressing retroviruses diffusely infect the optic nerve. A, the NICD1–3 and chimeric protein constructs. B, PLAP staining of the optic nerve of virally infected animals (5× magnification). C, i, RT-PCR showed that the Notch-expressing constructs increased Hes1 and Hey1 expression to similar levels in NIH3T3 cells when compared with CLE. ii, Notch-expressing constructs increased reporter expression from the Hes5promoter(Hes5p)-luciferase construct in E14.5 cortical neural progenitors. n = 3; *, P < 0.05.
identified, and these were limited to 2% to 4% of the eyes examined (Table 1). The generation of small glial proliferations by ΔN1CLE is consistent with a previous study showing that active Notch1 signaling promotes a glial cell fate in the developing retina, but failed to induce tumor formation within a 60-day postnatal period (28). ΔN2CLE caused the formation of cataracts, retinal disorganizations, and glial lesions in only 1% to 4% of the 113 eyes examined, but this may be due in part to the lesser extent of cells showing signs of viral infection.

**Analysis of receptor chimeras indicates a role for Notch3 TAD in glial tumor formation**

To address the mechanism underlying the dramatic phenotypic differences between the various Notch receptors, we generated a series of chimeras between NICD1 and NICD3. In brief, either the Notch3 TAD alone, or the Notch3 TAD with varying numbers of Ank were used to replace those of Notch1 (Fig. 4A). Interestingly, introducing the Notch3 TAD alone into NICD1 was sufficient to greatly enhance the number of glial lesions identified in vivo, suggesting that this region plays a key role in the differences between the 2 receptors (Table 1). It was also notable that none of our chimeric constructs were able to efficiently induce choroidal tumors. The only part of Notch3 lacking in all of these was the RAM domain, which mediates binding interactions between NICD and CBF1, thus this region may play an important role in the induction of this tumor type (Table 1).

As in the experiments above, infection of the optic nerve, retina, and forebrain, as visualized using the PLAP marker, was similar between chimeric constructs, suggesting that alterations in the number of infected clones did not account for the observed differences in phenotype (Fig. 5A). Additionally, the chimeric constructs were capable of activating the Hes5p- and CBF1RE-luciferase constructs in NIH3T3 cells, indicating these constructs were functionally capable of activating canonical Notch signaling (Fig. 5B).

**Notch activation does not drive glial tumor formation outside the optic nerve and retina**

We have previously shown that ΔN1CLE and N3CLE can promote astroglial fates in the postnatal forebrain (21, 22). Consistent with these prior observations, we found that the brains of ΔN1CLE-, ΔN2CLE-, ΔN3CLE-, and N3CLE-injected animals contained numerous PLAP-positive cells with an astrocytic morphology that were S100-immunopositive (Fig. 6A and B). Although some variation in the number of PLAP-positive cells was seen from animal to animal, the overall density of infected cells in the ΔN1CLE-, ΔN2CLE-, ΔN3CLE-, and N3CLE-injected cohorts was similar, although the affected region in ΔN2CLE-injected animals seemed more restricted to the ventral forebrain. As observed previously in N3CLE-injected animals, ΔN2CLE was able to induce choroid plexus hyperplasias or papillomas in 23 of 67 animals examined, and ΔN1CLE in 5 of 7 (Fig. 6C). The induction of papillomas by ΔN1CLE contrasts with our prior work, likely due to the use of the human receptor in this study as compared with the murine receptor previously (37).

Apart from ocular glial tumors, we did not identify glial tumors of the brain in any of the animals injected with active Notch1, Notch2, or Notch3. This implies that glial precursors in the optic nerve and retina have different tumorigenic potentials than those in the brain. To address whether this was due to clear differences in Notch pathway induction in the different tissues, we isolated neural progenitors from the optic nerve and cortex of newborn rat pups. Analysis of a variety of direct Notch pathway targets following infection with the CLE, ΔN1CLE, ΔN2CLE, ΔN3CLE, and N3CLE viruses revealed that signaling was induced in cells derived from both sites, but highlighted greater induction of Hes5 and Hey2 in the cortex as compared with the optic nerve (Fig. 6D).

**Discussion**

In this study, we show that the introduction of activated Notch3 into developing ocular tissues induced a number of aggressive neoplasms, including invasive pigmented tumors of the choroid of uncertain classification, and invasive gliomas arising from the optic nerve and retina. The neoplastic phenotypes observed in the eye are consistent with the known roles of Notch in ocular development. Notch2 and Notch3, and other pathway members, are expressed in the developing lens (29, 38). In the retina, Notch promotes progenitor cell character (30, 39), but it can also drive gliogenesis (40, 41). In both rodents and humans, astrocytic progenitor cells migrate during fetal and early postnatal life from the optic nerve into the retina and extend outward, forming a glial scaffold on which the retinal vasculature develops (42, 43). It has previously been
shown that Hedgehog signaling promotes the proliferation of optic nerve astrocytes (44), and our findings suggest that Notch may also play a role in this process and in the malignant transformation of astrocytes in these tissues.

A second significant finding in our study is that different Notch receptors do not have equivalent abilities to induce tumors in the optic nerve and eye. Only activated Notch3 was able to efficiently drive the formation of choroidal tumors and invasive glial lesions, whereas signaling by Notch1 and Notch2 generated no choroidal tumors and only very small glial lesions that did not have the capacity for diffuse invasion. These data are consistent with prior studies in which activation of Notch1 in the retina altered cell fates but failed to result in tumor formation (28, 30, 40, 41). Less is known about the roles of Notch2 and Notch3 in the developing eye and brain, although it seems in loss of function studies that Notch1 and Notch3 may play distinct roles in rod and cone differentiation (45).

To further investigate the molecular basis of the differences in tumor induction between Notch receptors, we generated and analyzed a series of chimeras using the intracellular domains of Notch1 and Notch3. These studies showed that replacement of the Notch1 TAD with that of Notch3 is sufficient to dramatically increase the formation of invasive glial tumors in vivo, and suggests that this domain plays a key role in specifying their disparate effects. Indeed, the largest difference between Notch1 and Notch3 is in the C terminus (21% amino acid identity), a region containing the TAD and PEST (Proline, Glutamine, Serine, Threonine rich motif) domains (36, 46). The TAD is associated with different post-translational modifications of the Notch receptors that may allow NICD to interact with other signaling pathways (47, 48). Early reports suggested that Notch3 lacks a TAD (46, 49) and that Notch1 is a much stronger activator of downstream signaling than Notch3 (46). More recent studies, however, have indicated that the spacing and orientation of binding elements in target promoters mediates some of these differences, and that the Notch3 TAD can efficiently activate the Hes5 promoter (19). Therefore, differences in Notch1 and Notch3 TAD structure and promoter binding element requirements may confer their differential tumorigenic potentials.

In addition to TAD, the RAM domain of Notch3 was also identified as a key region in the formation of choroidal tumors. The RAM domain mediates the binding of NICD to CBF1 (RBPjk in mice) and brings the Ank repeats close to their CBF1 binding sites. These protein–protein interactions are thought to recruit coactivator proteins, such as Mastermind (50), and displace corepressor proteins at the CBF1-transcriptional complex (51). Notch1 and Notch3 share low similarity in this region (41% amino acid identity; refs. 36, 46) and differences in the RAM domain may selectively modulate Notch activity.

Our final major finding is that glioma induction by Notch is dependent on the spatial location of neural stem/progenitor cells in which the pathway is activated. Despite roughly equivalent levels of viral infection in the optic nerve, retina, and brain, we only detected invasive gliomas in the first 2 structures. This was not due to a lack of virally infected...
glial precursors outside the optic nerve, as numerous PLAP-positive, S100β-positive astrocytic cells were present in brains injected with all 4 Notch-expressing viruses. In addition, no glioma induction in the brain was observed following introduction of NICD1 and NICD3 in earlier studies (21, 22, 52). Interestingly, Chambers and colleagues (53) have also shown that mammalian forebrain precursors have a variable response to Notch1 activation depending on spatial and temporal context.

These data raise the question of why glial precursors in the optic nerve and retina have a selective competence to form Notch3-induced tumors as compared with those in the brain. We examined the ability of the various Notch receptors to induce canonical downstream targets in optic nerve and brain-derived cultures, but did not find a lesser degree of activation in the brain-derived cells explaining their lack of neoplastic response (Fig. 6D).

In summary, our data expand the spectrum of solid cancers in which Notch activation alone is sufficient to induce tumorigenesis in vivo. They also support the concept that the same type of tumor in different locations along the neuroaxis might have distinct biologies due to inherently different properties of the cells of origin, a hypothesis recently advanced by a number of other groups (54–56). Finally, they indicate that the receptors Notch1, Notch2, and Notch3 are not equivalent in their ability to induce tumor formation in the brain, and suggest a role for the TAD in mediating these differences. Because selective targeting of individual Notch receptors in tumors might ameliorate treatment side effects, a better understanding of which receptor paralogs play critical roles in the initiation and growth of various cancer types is of clinical significance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Masoud Aghsaei-Fard and Dr. Katayoon Baradaran Ebrahimi for their help in isolating and characterizing rat neural progenitors and glia.

Grant Support

This work was supported by RO1 NS055089 (to C.G. Eberhart), Research to Prevent Blindness (to C.G. Eberhart), and RO1 NS046731 (to N. Gaiano).

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Received February 26, 2010; revised October 29, 2010; accepted November 24, 2010; published OnlineFirst January 18, 2011.

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Cancer Res  Published OnlineFirst January 18, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-10-0690

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2011/01/18/0008-5472.CAN-10-0690.DC1

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