

Tenascin-C Is a Novel RBPJ κ -Induced Target Gene for Notch Signaling in Gliomas

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Abstract

Tenascin-C (TNC) expression is known to correlate with malignancy in glioblastoma (GBM), a highly invasive and aggressive brain tumor that shows limited response to conventional therapies. In these malignant gliomas as well as in GBM cell lines, we found Notch2 protein to be strongly expressed. In a GBM tumor tissue microarray, RBPJ κ protein, a Notch2 cofactor for transcription, was found to be significantly coexpressed with TNC. We show that the *TNC* gene is transactivated by Notch2 in an RBPJ κ -dependent manner mediated by an RBPJ κ binding element in the *TNC* promoter. The transactivation is abrogated by a Notch2 mutation, which we detected in the glioma cell line Hs683 that does not express TNC. This *L1711M* mutation resides in the RAM domain, the site of interaction between Notch2 and RBPJ κ . In addition, transfection of constructs encoding activated Notch2 or Notch1 increased endogenous TNC expression identifying *TNC* as a novel Notch target gene. Overexpression of a dominant negative form of the transcriptional coactivator MAML1 or knocking down RBPJ κ in LN319 cells led to a dramatic decrease in TNC protein levels accompanied by a significant reduction of cell migration. Because addition of purified TNC stimulated glioma cell migration, this represents a mechanism for the invasive properties of glioma cells controlled by Notch signaling and defines a novel oncogenic pathway in gliomagenesis that may be targeted for therapeutic intervention in GBM patients. [Cancer Res 2009;69(2):458–65]

Introduction

Tenascin-C (TNC) is highly expressed in all major solid cancer types (for reviews, see refs. 1, 2). In most carcinoma cases, it is produced and localized in the tumor stroma, but the cancer cells themselves are mostly devoid of TNC (3, 4). This is, however, different in glioblastoma (GBM) where the highly invasive cancer

cells are the source of TNC themselves. The high expression of TNC in certain brain cancers was the basis for the original discovery of TNC as a glioma-mesenchymal extracellular matrix antigen (5). In the meantime, the presence of TNC in gliomas was found to correlate significantly with increased malignancy and poor clinical outcome of the disease (for review, see ref. 6). Immunohistochemical studies of gliomas have shown that TNC expression increases with tumor malignancy and function-blocking antibodies inhibited TNC-mediated proliferation and migration of GBM cells in culture (7). Furthermore, GBM patients with TNC immunopositivity in the tumor extracellular matrix had a significantly shorter survival compared with patients with GBM lesions lacking stromal TNC (8). It is, therefore, of interest to investigate the factors regulating TNC expression in GBM.

Notch signaling is an evolutionary conserved pathway that controls cell fate and growth. Ligand-dependent cleavage of the Notch transmembrane receptor releases the Notch intracellular domain into the cytoplasm. The intracellular domain translocates to the nucleus and binds and converts the transcriptional repressor RBPJ κ /CBF1/Su(H)/CSL to an activator by recruiting and binding coactivators such as mastermind-like proteins (MAML) to induce expression of Notch targets such as hairy/enhancer of split HES-1 and HES-5 in humans (9, 10). Notch2 is expressed during brain development in the cerebellar external granule layer and subventricular zones, where it maintains proliferation and prevents neuronal precursor differentiation (11). Notch2 expression in postnatal brain is restricted to ventricular germinal zones and dividing immature glial cells (12). Conversely, Notch2-deficient mice show increased apoptosis in neural tissues, leading to embryonic lethality (13). Thus far, oncogenic Notch2 functions have been reported in human B-cell leukemia (14) as well as in medulloblastoma (15). Notch1 oncogenic activity has been shown in gliomas (16) and other malignancies (17); however, it can also act as a tumor suppressor, depending on the cellular context (18).

Malignant gliomas are invasive tumors that can only transiently be controlled by surgery, radiotherapy, and chemotherapy (19–21). From a clinical point of view, conventional therapeutic interventions based on tumor resection and radiotherapy and chemotherapy have only moderately improved glioma patient survival over the past decades (22). Depending on histologic subtype, median survival of glioma patients varies between 10 months in the prevalent GBM and 10 years in low-grade oligodendroglioma (23). This is reflected in distinct pathogenetic pathways operative in these different glioma entities associated with variable degrees of

Note: B. Sivasankaran and M. Degen contributed equally to this work.

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doi:10.1158/0008-5472.CAN-08-2610

aggressiveness (21). Because we found in our previous study that loss of heterozygosity of *Notch2* is associated with prolonged survival of patients with oligodendroglioma or GBM with 1p loss (24), we investigated here whether Notch2 expression differs in oligodendroglioma versus GBM and whether Notch signaling could be linked to TNC expression.

Materials and Methods

Cell lines and antibodies. Cells were grown in DMEM with 10% FCS and 1% glutamine. Immunoreactions were performed with the monoclonal antibodies anti-Notch2 C651.6dbHN and anti-Notch1 bTAN20 (Developmental Studies Hybridoma Bank; ref. 25), anti-TNC B28-13 (26), anti-RBPJ κ (Institute of Immunology), anti-actin (Sigma), and antiserum against fibronectin (27).

Glioma biopsies and immunohistochemistry. Paraffin sections of formalin-fixed tissue samples were obtained from the University of Basel Department of Neuropathology in accordance with the guidelines of the ethical committee of the University of Basel. Tumors were diagnosed and graded according to the WHO Classification of Tumors of the Nervous System. Immunohistochemistry was performed with anti-Notch2 and anti-TNC using an overnight incubation at 4°C of a 1:50 dilution. Bound antigens were detected using avidin-biotin-peroxidase (Vectastain, Elite kit; Vector Laboratories), and sections were weakly counterstained with hematoxylin.

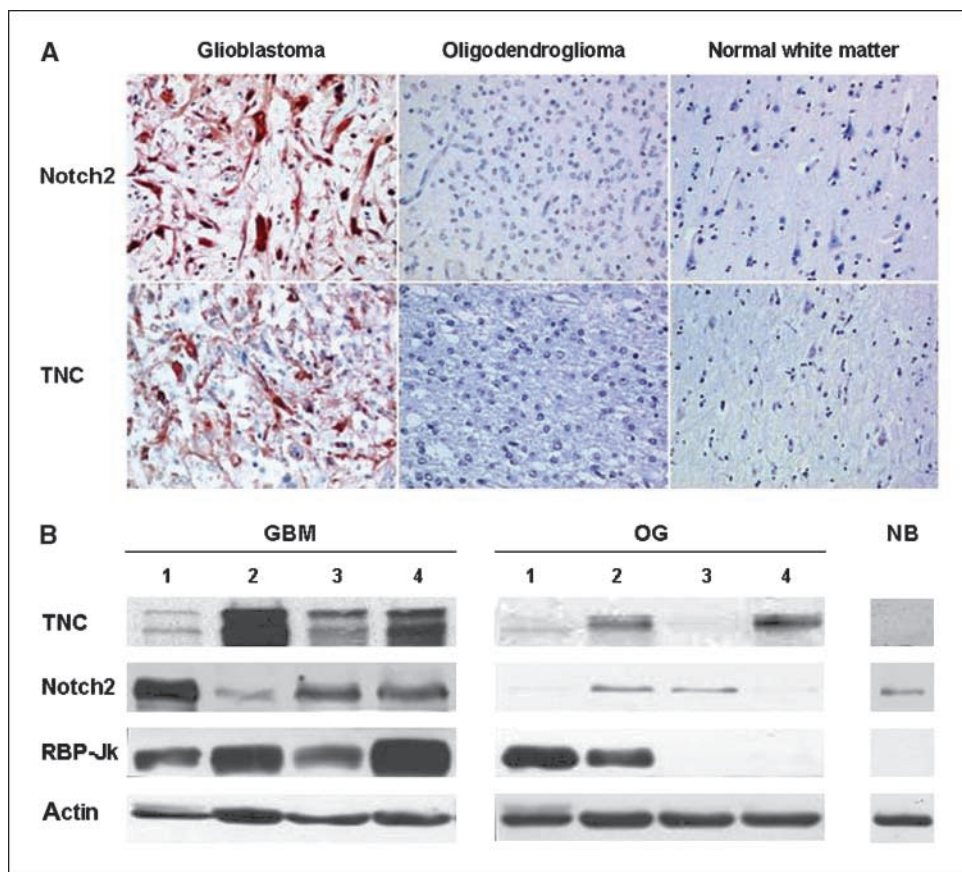
Tissue microarray. The tissue microarray comprising 190 GBM has been constructed from archived paraffin blocks at the University Hospital in Lausanne (28). Immunohistochemical determination for TNC (dilution, 1:2,500), anti-RBPJ κ (1:120), Notch2 (1:100), and Notch1 (Abcam; 1:200) was performed according to standard procedures for paraffin sections using a high-temperature epitope retrieval technique in citrate buffer (pH 6.0;

pressure cooker, 3–5 min) and overnight incubation with the primary antibody. The immunostaining was scored semiquantitatively by M-F. Hamou, who was blinded to the working hypothesis. In brief, the scores for TNC were as follows: 0, no staining; 1, partial or weak staining; 2, positive staining; 3, strong staining. Scores for Notch1 and Notch2: 0, no staining; 1, weak cytoplasmic and/or nuclear stain; 2, up to 50% positive tumor cells; 3, >50% positive tumor cells. Scores for RBPJ κ : 0, no staining; 1, few tumor cells with positive nuclei; 2, 15% to 40% of positive nuclei; 3, >40% of tumor cells with positive nuclei. Furthermore, scores included “ni,” which means not interpretable due to damaged or absent GBM tissue or unsuitable histologic area such as necrosis. Therefore, the number of interpretable GBM varied between the analyses. For pairwise comparisons (Fisher’s exact test, 2-sided), the scores were collapsed to low (score, 0–1) versus high (score, 2–3) expression, and excluding not interpretable samples. Tumors were ordered by similarity of expression profiles (scores, 0–3) using the SPIN software (29).

Nucleic acid extraction and analysis. Genomic DNA from glioma cell lines was extracted using the genomic DNA purification kit (Qiagen). RNA was isolated using Trizol (Invitrogen) reverse transcribed with the ThermoScript RT-PCR system (Invitrogen). Real-time quantitative PCR was performed on an ABI Prism sequence 7700 detector (PE Applied Biosystems) at microsatellite marker D1S2696 using primers GAATTA-CATCCAGGCAATCTGA and CACACAACAGGCCCTAATCA and probe FAM-AGCCCATGCTCATTCCCACTACTGG-TAMRA. *GAPDH* primers are AATGGGACTGAGGCTCCCAC and TTATGGGAAAGCCAGTCCCC and probe FAM-ATCCAAGACTGGCTCCTCCCTGCTG-TAMRA. *Notch2* cDNAs from glioma cells were sequenced at mutation hotspots (i.e., epidermal growth factor repeats 11–14, 24–25, 29 and 32; Lin-12 domains; and N2-IC). The Notch2 mutation L1711M found in Hs683 cDNA was confirmed by sequencing genomic DNA.

Western blot analysis. Cell extracts and conditioned media were separated by SDS-PAGE and electroblotted to polyvinylidene difluoride

Figure 1. Notch2 and TNC expression in GBM. A, immunostaining of GBM, oligodendroglioma, and normal white matter biopsies with anti-Notch2 and anti-TNC. Notch2 and TNC stainings were performed on sections of the same biopsies. B, immunodetection of TN-C, Notch2, RBPJ κ , and actin as loading control of tissue extracts of GBM, oligodendroglioma (OG), and normal brain (NB).



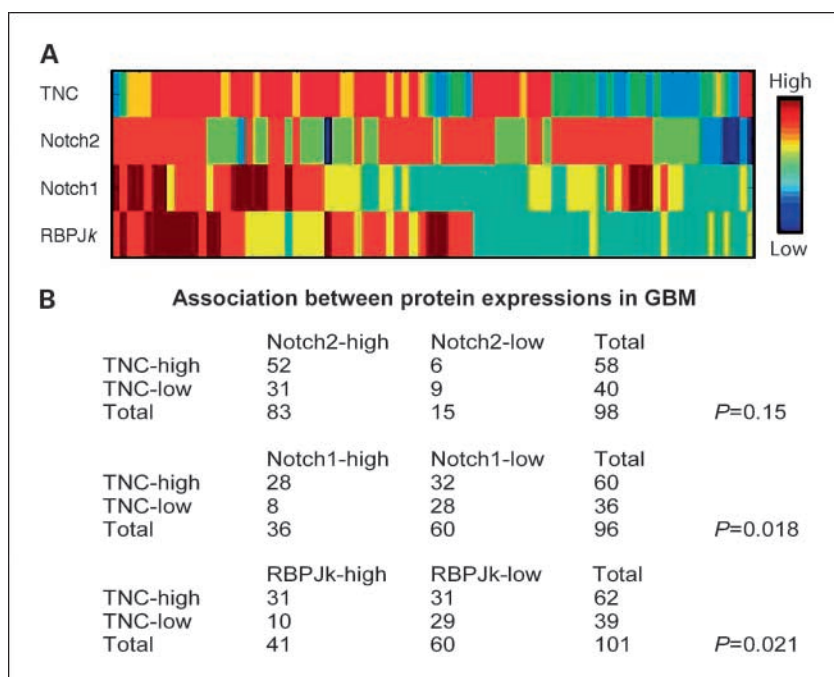


Figure 2. Notch1, Notch2, RBPJ κ , and TNC expression on a GBM tissue microarray. **A**, protein expression was determined by immunohistochemistry on a GBM tissue microarray and scored 0 (*indigo*, no expression) to 3 (*red*, high expression). Ninety-six of the 190 spots could be classified for all four proteins. Data are ordered using the SPIN software (35). **B**, associations between high and low expression levels of TNC with RBPJ κ , Notch2, and Notch1, respectively, were determined. Because noninterpretable spots varied on each section of the tissue microarray, the number of evaluable spots differed between each comparison. This analysis revealed a significant association between RBPJ κ and TNC levels ($P = 0.021$) and TNC and Notch1 ($P = 0.018$) but not for TNC and Notch2 ($P = 0.15$) as determined by the 2-sided Fisher's exact test.

membranes (Millipore). Proteins were detected with primary and secondary antibodies and blots were developed using Super Signal (Pierce) on Kodak BioMax MR Films. Protein bands were quantified using the software GeneTools (SynGene).

Plasmids and lentiviruses. Activated Notch2 (N2-IC, from nucleotides 5107–7425 of Notch2 cDNA sequence AF308601) was cloned into plasmid pcDNA3 (Invitrogen). Mutations in the Notch2-IC (L1711M) and in the RBPJ κ binding site (30) were obtained by site-directed mutagenesis (Stratagene). The Notch1 construct (31) was kindly provided by Dr. F. Radtke (Ludwig Institute for Cancer Research, Epalinges, Switzerland), the dominant negative RBPJ κ plasmid (32) was provided by Dr. T. Hanjo (Kyoto University Graduate School of Medicine, Kyoto, Japan), and the dominant negative MAML1 plasmid (33) was provided by Dr. J.C. Aster (Brigham and Women's Hospital, Boston, MA). The *TNC* promoter construct included 102-bp 5' flanking sequence from the transcription start and 97 bp of the first exon.

The reporter construct was cloned with the Expand High Fidelity PCR System (Roche) using the primer pair GAGCTAGCCTTAAATTCGC-CAACTG/GACTCGAGCTGCCTTTGTGCCACGGAG with genomic DNA from HEK293 cells as template and cloned into the pGL3 luciferase reporter vector (Promega).

The lentiviral vectors pLKO.1-puro, pLKO.1-scrambled-shRNA (Addgene), and pLKO.1-shRNA that targets RBPJ κ at 3-UTR (CCGGGCTGGAATA-CAAGTTGAACAACCTCGAGTTGTTCAACTTGTATTCCAGCTTTT) were transfected into HEK293 cells along with packaging plasmid (pCMV_dr8_91) and plasmid coding for envelope protein (pMD2-VSV-G). Concentration of infectious particles in the supernatant was titrated on HeLa cells.

Transfections and reporter gene assays. Transfections were performed using Fugene (Roche). For the reporter gene assays, Hs683 cells were seeded in 6-well plates and transiently transfected with 1 μ g TNC luciferase reporter vector, 1 μ g expression plasmid, and 0.1 μ g *Renilla* Luciferase reporter vector phRL-TK for standardization (Promega). Cells were harvested after 24 h and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured from at least three independent experiments in a Mithras LB940 Luminometer (Berthold Technologies). Western blots showing similar levels between N2-ICWT and N2-ICL1711M in transfected cells ruled out instability of N2-ICL1711M.

LN319 cells were transfected with the MAML1DN construct using CaCl₂ precipitation for 8 h. Stably transfected clones were selected for resistance to 100 μ g/mL Geneticin for 20 d. LN319 stably transduced with lentivirus constructs were selected for resistance to 0.5 μ g/mL puromycin.

Transwell migration assays. Transwell migration assays were performed using modified Boyden chamber units with polycarbonate filters of 8- μ m porosity (Costar). The lower side of the filter was coated with 10 μ g/mL fibronectin for 2 h at room temperature. The bottom chamber was filled with serum-free DMEM containing 0.1% BSA plus/minus increasing amounts of purified human TNC. Cells (10⁴ per well in serum-free DMEM) were plated in the upper chamber and incubated for 16 h at 37°C. After removal of the remaining cells from the upper surface of the filter, migrated cells at the bottom of the filter were fixed with 3.7% formaldehyde in PBS and stained with 0.1% crystal violet. For each condition, cells in five fields of three independent experiments were counted.

Statistical analyses. Kruskal-Wallis test was used for significance of genomic copy dosage. Significances of reporter gene expression and endogenous TNC expression were established with the Student's *t* test available at the Web server of St. John's University.

Results

TNC in gliomas correlates with Notch2 and RBPJ κ expression. In an initial immunohistochemical survey of glioma patients, we noticed strong Notch2 immunostaining in 2 of 2 astrocytomas grade II and 3 of 4 GBMs. In contrast, Notch2 protein was neither detectable in 6 of 6 oligodendrogliomas grade II and 2 of 2 oligodendrogliomas grade III, nor in normal brain tissue. We observed similar results for TNC in the same glioma biopsies and could correlate strong Notch2 staining in GBM sections with strong TNC immunopositivity. Examples are shown in Fig. 1A. This observation was confirmed by immunoblot analysis of TNC in brain tumor extracts. TNC expression was below detection in normal brain extract, low in oligodendrogliomas, whereas many high-grade astrocytomas and GBMs were rich in TNC (Fig. 1B). The median level of TNC in oligodendroglioma was 3.75 units

compared with 36.5 in astrocytoma and 56.2 units in GBM. Out of 19 GBM extracts analyzed, 17 (89.5%) showed higher TNC levels than any of the oligodendrogliomas; in 14 (82.4%) of these TNC-positive samples, Notch2 was detectable as well. Because canonical Notch2 signaling requires the presence of RBPJ κ , we determined whether this transcriptional regulator was coexpressed with TNC and Notch2. Indeed, there was a good correlation for the presence of all three proteins in GBM samples but not in extracts of oligodendrogliomas (Fig. 1B). Judging from the GBM immunoblots, it seems that the levels of TNC expression correlated better with RBPJ κ than with Notch2 levels (Fig. 1B, left). To extend our analysis, we made use of a GBM tissue microarray that allowed us to study a larger number of tumor samples; we also included Notch1 in our expression study (Fig. 2A). We obtained labeling frequencies of 83% (118 of 143) for Notch2, 59% (84 of 142) for Notch1, 37% (54 of 146) for RBPJ κ , and 55% (70 of 127) for TNC. Thus, Notch2 was more frequently detectable than Notch1. We found a significant association between RBPJ κ and TNC expression ($P = 0.02$) and Notch1 and TNC expression ($P = 0.02$). We also found a trend for an association between Notch2 and TNC ($P = 0.15$; Fig. 2B).

In a previous study, we have found that loss of a 1p centromeric marker within intron 12 of the *Notch2* gene was associated with favorable prognosis in oligodendroglioma as well as in GBM patients (24). Here, we show now that differing *Notch2* gene dosages are also found in glioma cell lines. Real-time PCR-based genomic DNA dosage analysis revealed that 4 of 8 GBM lines had amplification at the *Notch2* marker DIS2696, whereas LN18 cells exhibited local haploidy (Fig. 3A). Immunoblot analysis of Notch2 in these lines revealed concordance between genomic status and protein levels (Fig. 3B), suggesting that the genomic status at this locus may influence Notch2 protein levels. The glioma line expressing oligodendroglioma markers Hs683 contained only one *Notch2* gene copy per diploid genome. In all cell lines tested, we detected a striking coincidence between Notch2 and TNC protein levels, particularly visible between low Notch2 expression and absence of detectable TNC in Hs683 and LN18 cells (Fig. 3B). Finally, Notch1, Notch2, and RBPJ κ protein levels were compared with Notch signaling activity as assessed by *HES-1* transcript levels (Fig. 3C). RBPJ κ was present in all GBM lines, and decreased amounts of Notch1 or Notch2 were accompanied by lower *HES-1* expression, as can be seen in Hs683, LN18, and LN401 cells, indicating that there is a direct link between Notch protein levels and Notch signaling activities in the glioma cells analyzed.

The TNC promoter contains an RBPJ κ -responsive element.

The coexpression of TNC with Notch2 and RBPJ κ suggested the possibility of a causal relationship; therefore, *trans* activation of TNC by Notch/RBPJ κ signaling was considered. Alignment of the promoter sequences of the human and murine Notch target genes *HES-1* and *HES-5* with those of TNC revealed conservation of a perfect RBPJ κ binding motif GTGGGAA at the same distance from the TATA box in each of these genes (Fig. 4A). We therefore tested whether the intracellular domains of Notch2 (N2-IC) and Notch 1 (N1-IC) were able to induce luciferase reporter gene expression driven by a 102-bp fragment of a human *TNC* minimal promoter containing the RBPJ κ binding motif. Cotransfection of Hs683 cells with a plasmid expressing either N2-IC or N1-IC both led to a 2-fold increase compared with the basal luciferase activity in the presence of a control plasmid (Fig. 4B). To test whether this induction is dependent on RBPJ κ function, point mutations previously described to prevent RBPJ κ binding to target genes (30) were introduced into the potential RBPJ κ binding site of the

TNC promoter. Transfection of the mutant *TNC* promoter constructs M1 and M2 resulted in similar basal luciferase levels as the wild-type construct but no longer allowed induction by N2-IC (Fig. 4B). Consistently, cotransfection of a plasmid expressing a dominant negative RBPJ κ mutant (32) impaired N2-IC-mediated induction of the *TNC* promoter (Fig. 4B).

The TNC-negative Hs683 cells contain a Notch2 mutation.

We sequenced Notch2 fragments encoding the N2-IC and covering mutation hotspots for gain-of-function or loss-of-function described in *Drosophila* Notch (34), Notch1 (35), and Notch2 (36) in our glioma lines. Interestingly, the oligodendroglioma line Hs683

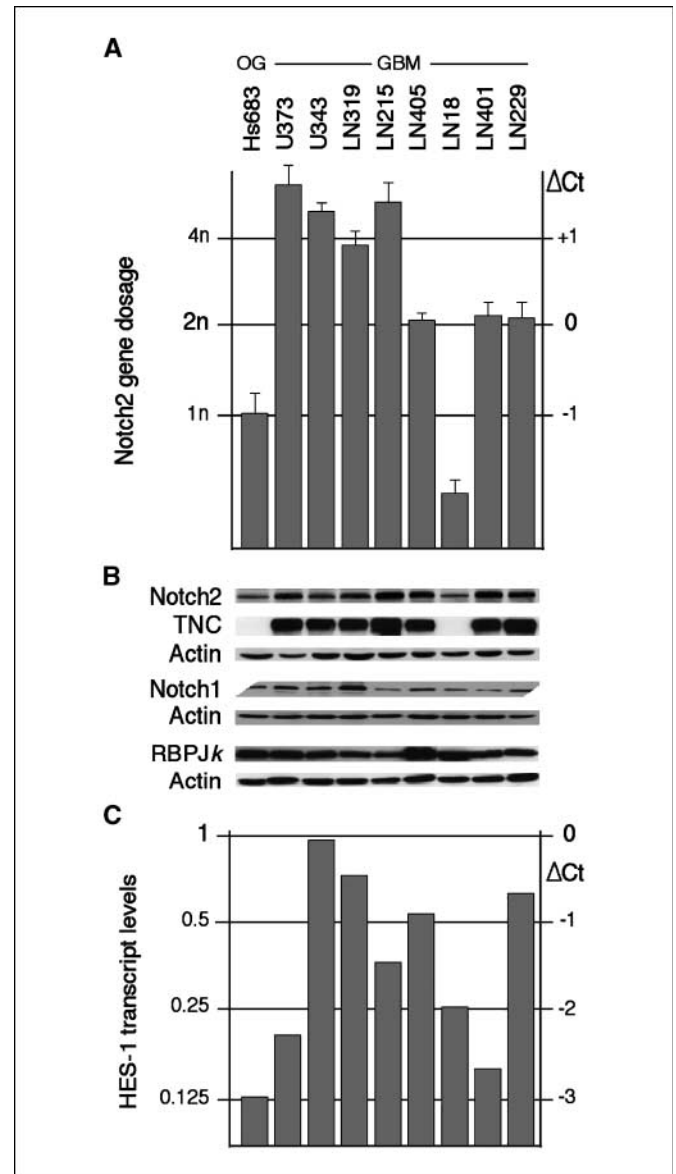


Figure 3. Notch genomic status and TNC expression in glioma cell lines. **A**, detection of genomic amplifications at marker DIS2696 in glioma lines by real-time quantitative PCR. A Δ Ct value of 0 corresponds to normal diploidy. Notch2 genomic status (n) is calculated as $2 \times 2^{-\Delta$ Ct}. All experiments were done in triplicates. Columns, mean based on two independent experiments; bars, SD. P value for comparison of all three groups ($>2n$, $2n$, and $<2n$) is 0.0002. **B**, Notch2, TNC, Notch1, and RBPJ κ immunodetection in glioma cells. For each experiment, the corresponding actin standard is shown below. **C**, assessment of Notch signaling activity in glioma cells by real-time quantitative PCR of *HES-1* transcripts. Δ Ct value of 0 corresponds to fetal brain level. *HES-1* amounts relative to fetal brain baseline calculated as 2^{Δ Ct}.

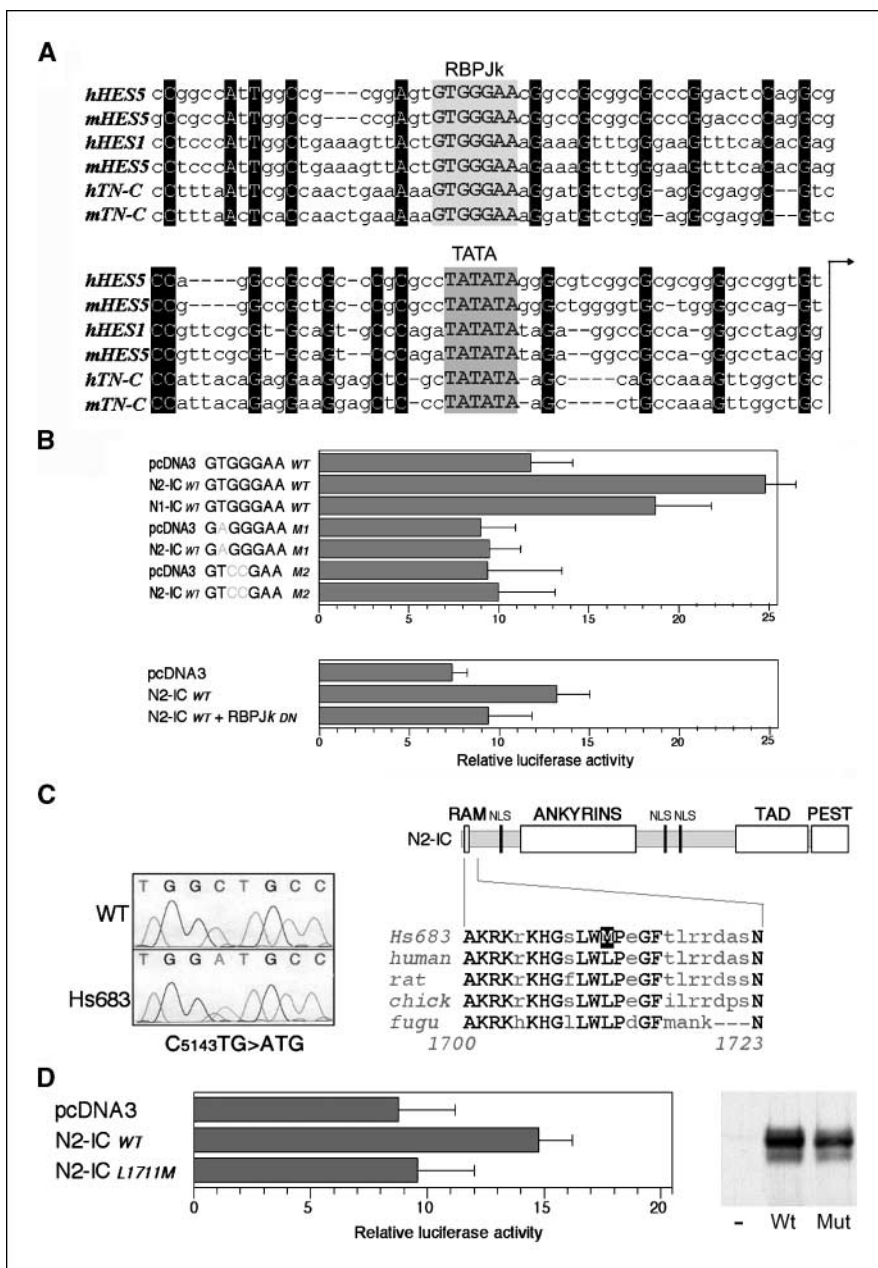


Figure 4. The *TNC* promoter contains a functional RBPJk responsive element. **A**, sequence alignment of human (*h*) and mouse (*m*) *HES5*, *HES1*, and *TNC* promoters. Conserved nucleotides are uppercase and highlighted in black. TATA boxes and RBPJk binding motifs are highlighted in gray. **B**, Notch-dependent induction of luciferase driven by the human *TNC* promoter occurs when the wild-type (WT) RBPJk binding site is present but not with *M1* or *M2* mutants. Columns, mean based on three independent experiments; bars, SD. Significance of N2-IC induction on GTGGGAA WT versus control pcDNA3 and *M1/M2* mutations: $P < 0.008$. A dominant negative (DN) RBPJk mutant impairs Notch2-dependent induction of *TNC* transcription. Significance of N2-IC induction versus pcDNA3 and RBPJk DN: $P < 0.008$; significance of N1-IC versus pcDNA3: $P < 0.035$ (bottom). **C**, electrophoretogram of the Hs683 mutation L1711M (left). Model showing the N2-IC domains: RAM, RBPJk-binding site; NLS, nuclear localization signal; TAD, trans activation domain; PEST, serine/threonine-rich domain. Below, a sequence alignment of the Hs683 Notch2 mutation with wild-type Notch2 RAM domains of several species shows high sequence identity highlighted in uppercase and bold and the mutation is highlighted. **D**, Notch2-dependent induction of the *TNC* promoter is impaired by the N2-IC L1711M mutation. Significance of N2-ICWT induction versus pcDNA3 and N2-ICL1711M: $P < 0.02$. An immunoblot of extracts of cells transfected with an empty pcDNA3 vector (-), the wild-type N2-IC (wt), or the mutated N2-ICL1711M (mut) shows that both Notch2 constructs gave comparable expression levels (right).

had a C-to-A mutation in Notch2 encoding leucine 1711, resulting in the substitution of this leucine by methionine (Fig. 4C). Leucine 1711 is conserved throughout vertebrate Notch2 proteins and is located within the RBPJk-interacting RAM domain. Coexpression of the N2-IC L1711M mutant with the *TNC* promoter construct no longer increased luciferase activity (Fig. 4D). In parallel, we performed immunoblots of cell extracts to verify that both the wild-type as well as the mutated N2-IC constructs resulted in similar expression levels (Fig. 4D). Therefore, Notch2, although present in the HS683 cells, seems to be nonfunctional.

Notch signaling regulates endogenous TNC expression. Next, we tested whether Notch signaling was able to induce the expression of endogenous *TNC* protein. Because the abundant *TNC* levels produced by GBM cell lines may mask any further increases, we focused on fibroblast lines Detroit 551 and MRC-5 that secrete moderate amounts of *TNC*. Supernatants of fibroblasts

transfected with N2-IC showed increased accumulation of *TNC* in the medium compared with the controls, whereas secreted fibronectin levels were similar (Fig. 5A). The same stimulation of *TNC* expression was detected after transfection of N1-IC but not with the mutant N2-IC L1711M construct (Fig. 5B). To further prove that *TNC* production was dependent on Notch signaling, we inhibited this pathway in LN319 cells, which express high levels of Notch1/2, RBPJk, and *TNC* (Fig. 3), by overexpression of a dominant negative form of MAML1 (33) or by down-regulation of RBPJk by shRNA (Fig. 5C). In both cases, a significant reduction of secreted *TNC*, with a more dramatic effect through MAML1 inactivation, was obtained (Fig. 5C). Thus, stimulating or blocking Notch signaling is accompanied by either up-regulation or down-regulation of *TNC*, respectively.

TNC enhances glioma cell migration. To test whether *TNC* expression plays a role in glioma cell migration, we performed

transfilter migration assays. Cell migration of the TNC-negative Hs683 cells could be stimulated by the addition of purified TNC to the bottom chamber (Fig. 6A). Because inhibition of Notch signaling by overexpression of dominant negative MAML1 in LN19 cells led to concomitant reduction of TNC expression, we compared cell migration of the parental versus the MAML1DN transfected LN319 cells. We found that cell migration was reduced in the MAML1DN transfectants to <50% of the parental cells

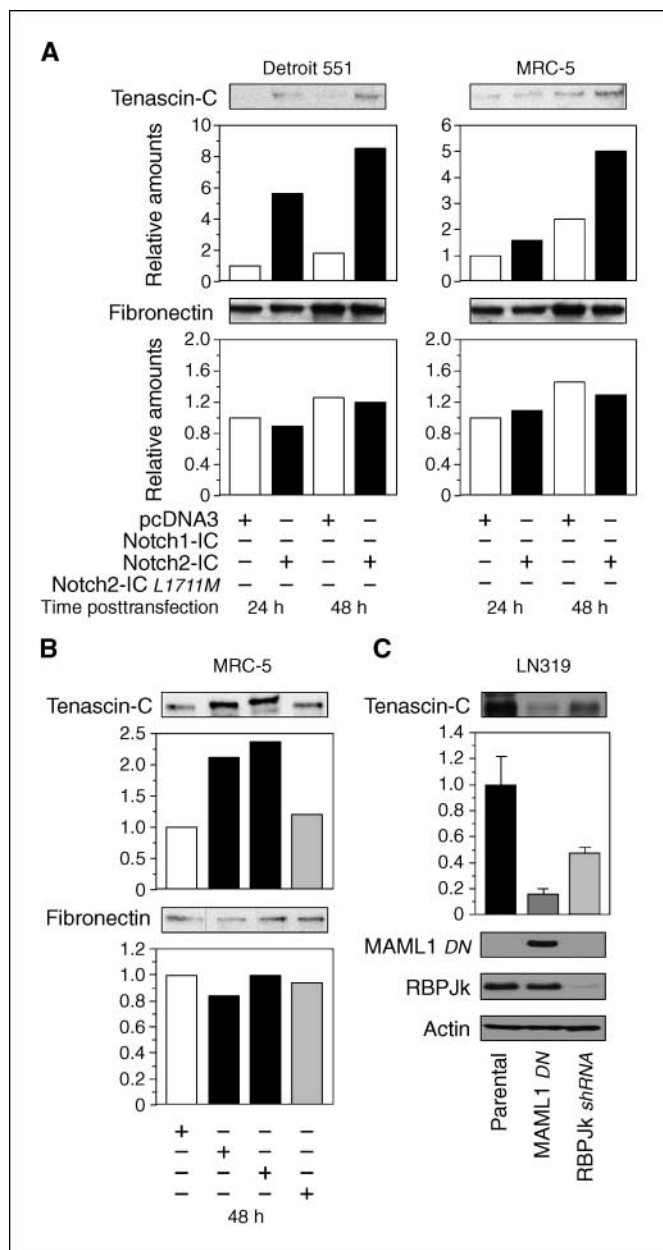


Figure 5. Notch signaling triggers endogenous TNC production. *A*, time course of Notch2-dependent endogenous TNC production. Detection of TNC and fibronectin on immunoblots of supernatants from transfected Detroit 551 and MRC-5 fibroblasts collected 24 and 48 h after transfection with Notch2-IC or the control plasmid pcDNA3. Graphs show quantification of TNC and fibronectin protein bands. *B*, transfection of wild-type Notch1-IC and Notch2-IC but not the mutated Notch2-IC L1711M lead to an increase of TNC levels in the medium of MRC-5 fibroblasts collected 48 h after transfection. *C*, disruption of Notch signaling results in lower TNC levels in extracts of LN319 cells. Signaling mediators MAML1 and RBPJ κ were genetically inactivated by stable expression of dominant negative MAML or RBPJ κ shRNA.

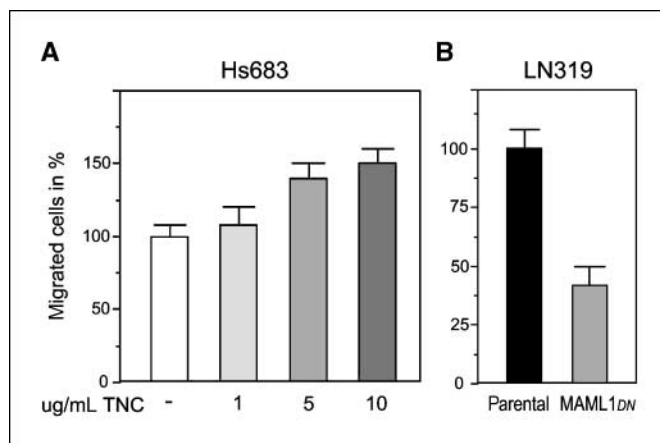


Figure 6. TNC promotes glioma cell migration. *A*, migration assays were performed in modified Boyden chambers with the TNC-negative Hs683 cells in the presence or absence of indicated amounts of purified TNC protein in the lower chamber. After 16 h, migrated cells were counted and numbers were normalized to Hs683 cells in the absence of TNC (set to 100%). Columns, mean based on three independent experiments; bars, SD. Significance of Hs683 versus Hs683 + 10 μ g/mL TN-C: $P < 0.001$. *B*, LN319 cells stably expressing dominant negative MAML1 (with inactive Notch signaling and reduced TNC expression) show reduced migration compared with the parental LN319 cells. Migration of the parental cells was set to 100%.

(Fig. 6B). Thus, Notch signaling directly regulates TNC expression, which in turn affects glioma cell migration. Because the cell migration capacity is required for the invasive behavior of glioma cells, our results provide a potential mechanism for oncogenic Notch signaling in gliomagenesis.

Discussion

Differential Notch2 expression in GBM and oligodendroglioma.

In this study, we found that Notch2 protein expression was much higher in GBM than in oligodendroglioma. The Notch2 loss-of-function mutation in Hs683 cells described in this article provides an inactivation mechanism of Notch2 in oligodendroglioma, in addition to homozygous deletions detected in primary oligodendroglioma with 1p loss described previously (24). Thus, oligodendroglioma development seems to depend on Notch2 reduction or loss.

In normal brain, Notch2 is expressed in immature glial cells of ventricular germinal zones (12, 37) and nestin, a marker for neural precursors, is regulated by Notch signaling (38). This implies that Notch2 may be involved in maintaining glioma cells in an undifferentiated state. The Notch target HES-1 drives an astrocytic over oligodendrocytic cell fate arguing for a role of Notch signaling in astrocytic differentiation (39). It has been shown that primary GBM shows a high HES-1 content, whereas astrocytoma progression coincides with decreasing HES-1 levels (40). Because HES-1 is under direct control of both Notch proteins, the question of whether Notch1 plays redundant or complementary roles to Notch2 in glioma progression remains open.

TNC is regulated by Notch signaling. Although Notch1 as well as Notch2 induced expression of TNC, a much larger number of GBM cases expressed high levels of Notch2 than Notch1. This high Notch2 expression may be based on the frequent *Notch2* gene amplification and high protein expression we found in GBM cells. The significant association between RBPJ κ and TNC expression on a GBM tissue microarray together with the identification of an

RBPJ κ -responsive element in a minimal *TNC* promoter provide a novel mechanism of *TNC* transactivation. Molecular cooperation between Notch2 and RBPJ κ has been suggested in B-cell development, where Notch2- and RBPJ κ -deficient mice present a common phenotype both lacking a B-cell subset (41), and in B-cell leukemia, where Notch2 and RBPJ κ up-regulate *CD23a* transcription (14).

Nevertheless, in half (31 of 62) of the TNC-positive GBM on the tissue microarray, RBPJ κ was not detectable, suggesting alternative RBPJ κ -independent regulatory pathways for TNC induction. In human fibroblasts, the *TNC* promoter is activated by transforming growth factor- β (TGF- β ; ref. 42). Because TGF- β signaling promotes PDGF- β -dependent cell proliferation in glioma (43), activation of the *TNC* promoter by TGF- β signaling may be an alternative pathway for TNC induction in glioma.

The Notch-TNC connection and invasion. TNC-deficient mice show compromised proliferation/migration of neural precursors and accelerated oligodendrocyte differentiation (44). Consistently, GBM lines and biopsies showed strong TNC expression, whereas oligodendrogliomas did not, except for an oligodendroglioma subset that has been described as moderately positive (45). Strong TNC expression is associated with the invasive front in many tumor types and is a diagnostic marker for glioma progression, implying a role for TNC in tumor promotion (for review, see ref. 2). These findings support the hypothesis that Notch2/RBPJ κ /TNC signaling is an important pathway operational in GBM but not oligodendroglioma development.

Our study presents the first description of an effect of Notch signaling on migration and invasion of glioma cells. Only very recently, reports started to appear describing a role of Notch signaling in invasion of mammary breast cancer cells to brain in a xenograft model (46), induction of snail and EMT in cultured cells

(47), or osteosarcoma invasion and metastasis to the lung in an orthotopic mouse model (48). Thus, a link between Notch signaling and invasion may become more generally recognized, and it will be interesting to investigate whether TNC is also involved as a mediator of the Notch effect in these other situations.

From a clinical point of view, conventional therapeutic interventions based on tumor resection and radiotherapy and chemotherapy have only moderately improved glioma patient survival over the past decades (22). In addition to direct targeting of TNC (49, 50), our data suggest the use of drugs blocking Notch signaling.

We propose that the Notch/RBPJ κ /TNC pathway regulates tumor cell migration, a hallmark of invasive GBM. This molecular cascade provides a novel mechanism through which Notch acts in neoplastic transformation and possibly in normal development of the neuronal and glial cell lineages.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/8/2008; revised 10/17/2008; accepted 10/20/2008.

Grant support: Krebsliga Beider Basel (7-2004; A. Merlo); OncoSuisse [OCS-01613-12-2004 (A. Merlo) and OCS01680-2-2005 (M.E. Hegi)]; Swiss National Science Foundation (3100AO-108266/1; M.E. Hegi); and Novartis Research Foundation (M. Degen and R.C. Ehrismann).

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We thank Beatrice Dolder and Elisabeth Taylor for outstanding technical assistance, Eytan Domany for the SPIN software, Juliane Hänggi for manuscript preparation, Freddy Radtke for the Notch1 construct, Tasuku Honjo for the dominant negative RBPJ κ plasmid, and Jon Aster for the dominant negative MAML1 construct.

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