Glioblastoma (GBM) is the most common malignant brain tumor that is characterized by high proliferative rate and invasiveness. Since dysregulation of Notch signaling is implicated in the pathogenesis of many human cancers, here we investigated the role of Notch signaling in GBM. We found that there is aberrant activation of Notch signaling in GBM cell lines and human GBM-derived neurospheres. Inhibition of Notch signaling via the expression of a dominant negative form of the Notch co-activator, mastermind-like 1 (DN-MAML1) or the treatment of a γ-secretase inhibitor (GSI) MRK-003 resulted in a significant reduction in GBM cell growth in vitro and in vivo. Knockdown of individual Notch receptors revealed that Notch1 and Notch2 receptors differentially contributed to GBM cell growth, with Notch2 having a predominant role. Furthermore, blockade of Notch signaling inhibited the proliferation of human GBM-derived neurospheres in vitro and in vivo. Our overall data indicate that Notch signaling contributes significantly to optimal GBM growth, strongly supporting that the Notch pathway is a promising therapeutic target for GBM.

Keywords
Notch signaling; Glioblastoma; Tumor neurospheres; γ-secretase inhibitor; Cell growth
Introduction

Glioblastoma (GBM) is the most common malignant brain tumor and is characterized by high proliferation rate and invasiveness. The median survival of GBM patients ranges from 9–15 months, even with use of the most aggressive treatments which include surgery, radiation, and chemotherapy (1–4,5). Dysregulation of a number of signaling pathways have been reported in GBM pathogenesis. These pathways include cell cycle control (such as P16\(^{\text{INK4A}}\)-CDK4-RB, P18\(^{\text{INK4C}}\), p19\(^{\text{ARF}}\)-MDM2-p53 pathways), growth factor receptors (such as platelet-derived growth factor receptor, epidermal growth factor receptor), transforming growth factor \(\beta\) signaling, and phosphoinositide 3-kinase/phosphatase and tensin homolog (PTEN) signaling (3,4). Signaling pathways, regulating stem cell development may also play a role in the pathogenesis of this disease as cancer stem cells have been isolated from GBM (6–11). A further understanding of these signaling pathways may lead to novel therapies and strategies which will advance the treatment of GBM.

Notch signaling is an evolutionarily conserved pathway that plays an important role in multiple cellular and developmental processes. These processes include cell fate decision, differentiation, proliferation, survival, angiogenesis, migration and epithelial-mesenchymal transition in many tissues, including brain (9,12–14). There are four Notch receptors (Notch1-4) and five ligands (Jagged1, Jagged2 and Delta-like 1, 3, and 4) identified in mammals (12,13). Notch receptors are conserved type I trans-membrane receptors. The binding between Notch receptors and ligands on neighboring cell leads to multiple steps of proteolytic cleavage of the receptors and releases the Notch intracellular domain (NICD), which translocates into nucleus. In the nucleus, NICD binds to transcription factor CSL (\(C\) promoter binding protein-1 in mammals, Suppressor of Hairless of Drosophila, \(\text{Lag}-1\) in \(C\). elegans) and the co-activator mastermind-like proteins (MAML1-3), initiating transcriptional activation of Notch target genes (13–16). Dysregulated Notch signaling has been implicated in many tumors including hematological cancers, such as T-cell acute lymphoblastic leukemia (T-ALL), Hodgkin’s lymphoma, some of the acute myeloid leukemias, multiple myeloma and solid tumors, such as glioma, cervical, lung, pancreatic, breast cancer and hepatocellular carcinoma (13,15,17–19).

Activated Notch signaling can be either oncogenic or tumor suppressive depending on the cellular and physiological context (13,15,20). In normal brain development, the Notch pathway is known to have an impact on neural stem cell renewal, progenitor cell differentiation, learning, memory and gliogenesis (21–24). In brain cancer development, Notch1 was previously reported to be oncogenic (25). Notch1 and its ligands Delta-like-1 and Jagged1 were reported to be crucial for GBM cell growth (26). Notch1 and Notch2 were shown to have opposite effects on medulloblastoma growth (27). Loss of Notch2 was a predictor of survival in subgroups of human glial brain tumors (28). Recently, tenascin-C, an extracellular matrix protein with a role in migration, was identified as a Notch target gene in gliomas (29). Although increasing evidences suggest that Notch signaling plays a role in GBM pathogenesis, the mechanisms underlying Notch activation and the importance of specific Notch receptor(s) in the regulation of GBM growth remain poorly defined.

Recently, cancer stem cells were identified in GBM (6–8,10,11,30). Notch signaling was also implicated in neural stem cell renewal, proliferation and differentiation (21,22). Further investigation into the role of Notch signaling in GBM cancer stem cell development will provide new insights into the pathogenesis of this disease. Targeting these cancer stem cells may prevent the predictable recurrence of GBM (7).
In this study, we determined the role of Notch signaling in established GBM cell lines and in human GBM-derived neurospheres. We observed elevated Notch signaling in GBM, which is shown by the enhanced expression levels of ligands, receptors and target genes from our analysis of data from Serial Analysis of Gene Expression (SAGE) database. We then validated the expression levels of Notch signaling components in GBM cells and tumor neurospheres by Western or quantitative RT-PCR analysis. We also investigated the importance of Notch signaling and individual Notch receptors in the growth of GBM cell lines and tumor neurospheres in vitro and in vivo. Our overall findings revealed a crucial role of Notch signaling activation in the pathogenesis of GBM.

Results

GBM cell lines express high levels of Notch signaling components

Notch signaling was shown to be involved in the pathogenesis of many tumor types (15,17). Therefore, we used an unbiased approach- SAGE analysis- to determine the relative expression levels of the Notch pathway components across different tumor types compared to their normal cognate tissues, and to determine whether the expression of Notch components correlates with tumorogenicity (31). After analyzing the expression of Notch ligands, receptors and Notch target genes in seventeen tumors, we found elevated expression of DLL1, DLL3, NOTCH1, NOTCH2, HES1, HES5, HEY1, DTX2 and NRARP in brain tumors as compared to normal brain samples (Supplementary Figure 1 A and Supplementary Table 2). As the brain tumor samples contain 6 different subtypes (28 medulloblastoma, 20 astrocytoma, 19 glioblastoma, 12 ependymoblastoma, 5 meningioma, 2 oligodendroglioma and 8 normal brain samples), we analyzed the expression levels of Notch signaling components in each of the subtype compared to normal brain (Supplementary Figure 1 B and Supplementary Table 3). We found significant elevation of Notch signaling gene expression in five out of six sub-types, with astrocytoma having elevated expression of DLL3, NOTCH1, NOTCH2, HEY1, HEY2; ependymoblastoma having increased expression of NOTCH2, HES1, HEY1; GBM having elevated NOTCH2, HES1 expression; oligodendroglioma having increased HES1, HEY1 and DTX1 expression; while meningioma having elevated HES1 expression. Since GBM is the most malignant among brain tumors and has significantly increased levels of the Notch signaling-related gene expression by SAGE analysis, we used five GBM cell lines LN827, deltaU87, U87, U251 and LN428 to further test and confirm the expression levels of the Notch pathway components. All these five cell lines expressed Notch1, 2, 3 RNA and protein (Figure 1A, B). Compared to NOTCH1 and 3 transcripts, the RNA level of NOTCH2 was 10 and 20 fold higher respectively, while NOTCH4 transcripts were undetectable in these cell lines (Figure 1A). All five cell lines express JAGGED-1 and JAGGED-2 protein, with U251, LN428 having the highest amounts (Figure 1B). DELTA-LIKE-1, DELTA-LIKE-3 and DELTA-LIKE-4 are undetectable in these cells (data not shown). All five cell lines expressed Notch target genes HES1, HEY1 and C-MYC proteins (Figure 1B). LN428 appears to have higher NOTCH1 expression at the protein level than the RNA level, which is likely due to the translational regulation of Notch1 by Ras and Akt pathway as reported previously (32). These data indicated that Notch signaling is activated in GBM, which may play a role in the pathogenesis of this tumor. Recently, Notch and Sonic hedgehog signaling were reported to be highly expressed in the Classical subtype of GBM (33), further highlighting the clinical relevance of Notch signaling in GBM.

Inhibition of Notch signaling by the dominant negative form of mastermind-like 1 significantly reduced GBM cell growth in vitro and in vivo

Since we found that Notch signaling is highly expressed in GBM, we next determined whether it is important for GBM cell growth. We blocked Notch signaling in five GBM cells
by transducing cells with lentiviruses expressing a dominant negative form of Notch co-activator mastermind-like1 (DN-MAML1) (Supplementary Figure 2) (34) and monitored cell growth. The expression of DN-MAML1 strongly inhibited Notch signaling, as there were a 60%–90% reduction of Notch target gene C-MYC expression in all five cell lines, a 56%–67% reduction of HEY1 expression in LN827, deltaU87 and U87 cells, and a 60%–80% of reduction of HES1 expression in deltaU87, U87, U251 and LN428 cells transduced with DN-MAML1 virus compared to cells transduced with GFP control virus (Figure 2A). Importantly, cells with DN-MAML1 expression showed reduced growth in LN827, deltaU87, U87, U251 and LN428 cells by 84%, 72%, 58%, 59% and 30% respectively on day 5 or day 6 post-transduction compared to cells transduced with control virus (Figure 2B). A time course of GBM cell growth showed that the growth was inhibited 2–29 fold by DN-MAML1 on day 8 or day 9 post-transduction (Supplementary Figure 3). These results indicated that inhibition of Notch signaling significantly reduced GBM cell growth in vitro. To evaluate the functional significance in vivo, the most Notch responsive cell line, LN827 cells were transduced with DN-MAML1 or GFP control viruses and then the infected cells were injected intracranially into SCID mice. Mice injected with DN-MAML1 transduced LN827 cells had significantly longer survival compared to those with GFP control virus transduced cells (p=0.0006) (Figure 2C (a)). The tumors were excised from the mice at sacrifice and the RNAs were extracted. Comparing to GFP controls, the tumors generated by DN-MAML1 infected cells have a significant reduction of C-MYC, HES1 and HES5 expression (Figure 2C (b)). Taken together, these data demonstrated that inhibition of Notch signaling by DN-MAML1 significantly reduced GBM cell growth in vitro and in vivo.

The expression of the dominant negative form of mastermind-like 1 caused G0/G1 cell cycle arrest and induced apoptosis in GBM cell lines

Since expression of DN-MAML1 caused growth suppression of GBM cells, we further determined the molecular mechanisms for cell growth inhibition by analyzing cell cycle profile and cell apoptotic rate. We observed that lentiviral-mediated DN-MAML1 expression in LN827 and deltaU87 cells led to an increase of cell populations in the G0/G1 phases, as compared to control GFP expression (65.88% vs. 47.01% in LN827) and (85.46% vs. 60.13% in deltaU87) respectively (Figure 3A). Forty-eight hours of DN-MAML1 expression in LN827, deltaU87 and U87 cells led to a significantly higher number of apoptotic cells compared to GFP control cells (Figure 3B). As CYCLIN D1 is a known Notch target gene (35) that plays an important role in cell cycle progression in G1 phase (36, 37), we measured the CYCLIN D1 expression levels by qPCR and Western analysis. We found that DN-MAML1 expression reduced CYCLIN D1 expression (Figure 3C and Supplementary Figure 4 C (a)), which might contribute to increased cell populations in the G0/G1 phases. Our data therefore indicated that dominant negative Notch inhibitor DN-MAML1 caused cell growth suppression in part by down-regulating CYCLIN D1 expression, inducing cell cycle arrest and apoptosis.

γ-secretase inhibitor MRK-003 inhibited Notch signaling, reduced cell growth and induced apoptosis in GBM cells

Activation of Notch signaling involves a critical step of proteolytic cleavages upon ligand binding. The cleavage of Notch receptors by γ-secretase protein complex releases the Notch intracellular domain, which then translocates to the nucleus and activates Notch target genes. We used the γ-secretase inhibitor (GSI) MRK-003 to test whether inhibition of Notch signaling at the step of receptor processing would also inhibit GBM cell growth. GSI effectively inhibited Notch signaling as shown by down-regulation of C-MYC and HES1 expression in a dose dependent manner (Figure 4A). Consistent with the decrease in C-MYC RNA levels, we also observed a decrease of C-MYC protein level after 24 hours of 10 μM and 20 μM GSI treatment (Supplementary Figure 4 A). The GSI effect is specific for Notch
signaling inhibition, since the phosphorylation status of other signaling pathways (including phospho-AKT of the PI3-kinase pathway or phospho-pp44/42 of the MAP-kinase pathway) was not affected (Supplementary Figure 4 B). GSI treatment also decreased CYCLIN D1 RNA levels and protein levels, which could contribute to G0/G1 cell cycle arrest (Figure 4B and Supplementary Figure 4 C (b)). Furthermore, GSI inhibited all five GBM cell line growth in a dose-dependent manner (Figure 4C). The IC50 for cell growth in these cells varied between 7–16 μM. LN827, deltaU87 and LN428 were the most sensitive cell lines as their growth was inhibited 50–80% at 10μM and 100% at 20μM and was consistent with decreased level of CYCLIN D1 expression (Figure 4B). Since the effects of Notch inhibitors on cell growth were profound, we further tested the effects of MRK-003 on GBM cell survival. Twenty-four and 48 hours after MRK-003 treatment, GBM cells underwent significant apoptosis compared to DMSO control treatment (Figure 4D). These results demonstrated that inhibition of Notch signaling by GSI treatment causes GBM cell growth suppression via inducing cell cycle arrest and apoptosis, strongly supporting an important role for the Notch pathway in GBM cell growth.

**Notch1 and Notch2 have differential effects on GBM cell line growth**

These GBM cells express Notch receptors 1, 2 and 3, with NOTCH2 being the most abundant. NOTCH2 mRNA was 10-fold higher than NOTCH1 and 20–25 fold higher than NOTCH3 in the cells studied here (Figure 1A). To further investigate the importance of each receptor in GBM cell growth, we knocked down Notch1, Notch2 and Notch3 (not shown) using shRNAs. The knocking-down of each Notch receptor was specific (i.e. the protein levels of each Notch receptor were not altered by the knockdown of the other two) (Figure 5A). Comparable down-regulation of NOTCH1 or NOTCH2 in deltaU87 and U251 cells significantly reduced HEY1 expression compared to scramble control (Figure 5B). We observed that NOTCH1 knockdown resulted in a reduction in cell growth (60%–80% decreased growth in LN428 and U251, respectively), as compared to control cells (Figure 5C (a)). Notch2 knockdown with two shRNAs significantly inhibited the growth of all five cell lines 50%–70% compared to scramble control infected cells (Figure 5C (b)). Notch3 knockdown only had minor effect on U251 and LN428 cell growth, but not the other three cell lines (data not shown). Therefore, these data indicate that both Notch1 and Notch2 are involved in regulating GBM cell growth while Notch2 has a more dominant effect on these cells.

**Inhibition of Notch signaling reduced human GBM neurosphere growth in vitro**

Since inhibition of Notch signaling by either DN-MAML1 or GSI prevented GBM cell growth in vitro and in vivo, we further tested whether the Notch signaling inhibition could affect the growth of human GBM neurospheres. These neurospheres were derived from primary GBM tumors and cultured in the stem cell medium. Compared with serum cultured cell lines, these neurospheres harbor more authentic tumor features and thus provided as a useful tool to study the biology of GBM (30). Some neural stem cell markers, such as CD133, NESTIN and ALDH1A were expressed differentially among these neurospheres, indicating the neural stem cell features of these spheres (Supplementary Figure 5). Furthermore, all three neurosphere lines express Notch ligands, receptors and target genes at relative high levels, suggesting that they harbor intact Notch signaling and activity (Supplementary Figure 6). Expression of DN-MAML1 in neurosphere lines BT70, BT74 and BT37 resulted in a significant down regulation of C-MYC, HES1 and HEY1 expression compared to GFP control, indicating that these neurospheres responded well to Notch inhibition (Supplementary Figure 7). DN-MAML1 inhibited sphere formation of BT70, BT74 and BT37 lines by 29%, 39% and 30% respectively compared to GFP control virus infected neurospheres (Figure 6A). This observation was consistent with study form Fan et al. reported that Notch pathway blockade depletes CD133-positive GBM cells and
neurosphere growth (38). We also used GSI to further confirm this growth inhibition effect. BT37 neurosphere was chosen as it has the most Notch target gene down-regulation after DN-MAML1 expression (Supplementary Figure 7). GSI inhibited BT37 neurosphere formation in a dose dependent manner. Treatment of 10μM MRK-003 inhibited 30% of neurosphere formation in 5 days, while treatment of 20μM MRK-003 inhibited almost 100% of neurosphere formation compared to DMSO control (Figure 6B (a) (b)).

**Inhibition of Notch signaling prevented GBM neurosphere growth in vivo**

To determine whether the in vitro inhibition of Notch signaling has functional relevance in vivo, equal numbers of BT37 neurosphere cells, pretreated with 10μM GSI or the DMSO vehicle control for 24 hours, were intracranially injected into SCID mice. We found that mice injected with BT37 cells pretreated with GSI had significantly prolonged survival ($p=0.0348$) compared to the controls (Figure 6C). These data indicated that inhibition of Notch signaling prevented GBM neurosphere growth in vivo (Figure 6C).

**Discussion**

The Notch signaling pathway has been associated with the pathogenesis of many cancers (15). Here, we investigated the importance of Notch signaling and different Notch receptors in GBM, the most malignant type of brain cancer. After identifying elevated Notch signaling using web-based SAGE analysis, we confirmed the expression of Notch receptors 1,2,3, Notch ligands Jagged1 and Jagged2 in GBM cells, suggesting possible juxtacrine or autocrine models of Notch activation. In addition, Notch signaling components were expressed in human GBM-derived neurospheres. These findings were consistent with other previous reports (25–28), showing Notch signaling components were expressed in different brain tumors and play important roles in their pathogenesis. Recently, Verhaak et al, reported that Notch signaling was highly expressed in the Classical subtype of GBM (39). We further showed that DN-MAML1 and GSI inhibited Notch signaling and differentially inhibited GBM cell growth. The difference of growth inhibition could be due to the different cellular context and heterogeneous genetic background of these GBM cell lines. For example, U251 and LN827 express p53 mutants, while U87 and deltaU87 cells contain wildtype p53 (40). LN428 cells harbor wild-type PTEN while the other four cell lines are PTEN null (40)(data not show). PTEN mutation renders T-ALL cells resistant to Notch inhibition by GSI treatment (41,42), as aberrant PI3-kinase/Akt signaling contributes to cell growth and survival. Whether mutation in PTEN contributes to GSI resistance in GBM cells need to be investigated further.

Involvement of Notch signaling in cancer is highly context dependent. In embryonal brain tumors, Notch1 inhibits while Notch2 promotes tumor growth (27). In GBM, Notch1 and its ligands Delta-like-1 and Jagged1 has been reported important for tumor cell survival and growth (26). Also, loss of Notch2 positively predicts survival of human glial brain tumors (28). Our results demonstrated that Notch2 is the predominant Notch receptor that contributes to the growth of all five GBM cell line tested; while Notch1 contributed to U251 and LN428 cells. It is possible that as the RNA expression of NOTCH2 is ten times higher than NOTCH1 in these GBM cells, they have more active Notch2, and thus are more dependent on Notch2 for growth. U251 and LN428 cells have relatively high protein levels of Notch1, and ligands Jagged1, Jagged2. Therefore, these two cell lines may have more active Notch1 than other three cell lines, which render them dependant on Notch1 for growth as well. Our results suggest that Notch2 and Notch1 are important for optimal GBM cell growth and therefore represent potential therapeutic targets. It would be interesting to identify the additional downstream target genes of Notch2 and Notch1 that facilitate their oncogenic functions. C-MYC is one of the interesting downstream candidates as it is a direct target of Notch signaling and has an oncogenic role in many tumors (43,44).
As GBM is one of the most malignant brain tumors, the most effective treatment so far has been surgery followed by radiation with concurrent and adjuvant temozolomide (TMZ) treatment. Invariably all GBM patients succumb to recurrence within a few years. The resistant nature of GBM may be due to the fact that the GBM stem cells are resistant to this standard treatment (45,46). We demonstrated that inhibition of Notch signaling by DN-MAML1 and GSI prevented GBM-derived neurosphere growth in vitro and in vivo. We also found GSI sensitized GBM cell lines and neurospheres to TMZ treatment and radiation (our unpublished data), which was reported by Wang et al., (47). Therefore, targeting GBM by Notch inhibition together with chemotherapy and radiation might lead to more durable clinical outcomes. It would be of great interest to further characterize the down-stream molecular targets of Notch signaling that play roles in the renewal and proliferation in GBM and cancer stem cells.

The results presented here may have clinical relevance in light of a recent report of a phase I clinical trial of GSI (MK-0752) in advanced cancers which showed a partial responder in malignant glioma (51).

In addition, the dominant negative form of MAML1 was found to have significant impact on GBM cell line and tumor neurosphere growth. Since a cell-permeable, stabilized peptide that can directly inhibit Notch signaling is now available (33), it will be of great interest to further test the effect of this peptide inhibitor on GBM cell line and tumor neurosphere growth, as it is a potential therapeutic tool with less off target toxicity.

In summary, we demonstrated that Notch signaling is a major contributor for GBM cell growth. Notch2 and Notch 1 are important Notch receptors that mediate this growth requirement, with Notch2 plays a more predominant role. Moreover, inhibition of Notch signaling by DN-MAML1 or GSI prevented human GBM-derived neurosphere growth. Our data suggest that inhibition of Notch pathway is a promising therapeutic approach for GBM.

**Material and Methods**

**Serial Analysis of Gene Expression (SAGE) analysis**

Web-based tool SAGE Anatomic Viewer (http://cgap.nci.nih.gov/SAGE/AnatomicViewer) was used to obtain the relative expression of sixteen Notch signaling genes including 5 Notch ligands, 4 Notch receptors and 7 known Notch target genes in a panel of 17 tumors and their cognate normal tissues. Each tumor type and its corresponding normal tissue were composed of samples (1–94) from the SAGE library. These samples are either patient bulk tumors or established human cell lines. GeneBank accession number of each Notch signaling gene was inputted and the short tags with the highest rank were used to blast the SAGE automatic viewer (Supplementary Table 1). Mean of the tags per 200,000 which represents the expression of a gene in each tumor and its cognate normal tissue was obtained. The expression fold change of the gene was obtained by dividing the mean of the tags per 200,000 of tumor over normal (Supplementary Table 2). As the brain tumor cohort has 94 samples (28 medulloblastoma, 20 astrocytoma, 19 glioblastoma, 12 ependymoblastoma, 5 meningioma, 2 oligodendroglioma and 8 normal brain samples), we compared the tags per 200,000 of Notch signaling genes in each brain tumor subtype to normal brain tissue to delineate the Notch expression (Supplementary Table 3). The fold change was transformed to LOG (2) value. If the tags per 200,000 of a gene in one tissue was zero (no expression), 1 was used to replace 0 when transforming into the LOG (2) value. The values were subjected to Hierarchical clustering (average linkage analysis) using the DNA-Chip Analyzer software (www.dchip.org).
Plasmids and small hairpin RNAs
The FLAG tagged 1-302aa MAML1 (34) was cloned into the NheI site of lentiviral vector pCSGW2-RES-GFP. Small hairpin RNA (shRNA) lentiviral constructs targeting Notch1 (#a clone Id TRCN0000003359, #b TRCN0000003362) and Notch2 (#a clone Id TRCN0000004895, #b TRCN0000004896) were obtained from Dana-Farber RNAi screening facility (Dana-Faber Cancer Institute, Boston, MA). pKLO.1-scramble control vector was obtained from Addgene (Addgene, Cambridge, MA). PMD2-VSV-G and pCMV_dr8.91 were gifts from Dr. William Hahn (Dana-Faber Cancer Institute, Boston, MA). Hairpin sequence for TRCN0000003359:
CCGGCTTTGTTCAGTATCTCAGAATACTGAACCTGAAACAAAGTTT
TT, Hairpin sequence for TRCN0000003362:
CCGGCAAAGACATGACCAGTGGCTACTCGAGTAGCCACTGTCTCTTTTT
TT, Hairpin sequence for TRCN0000004895:
CCGGGCAAGAATTGTCAGACAGTATCTCGAGATACTGTCTGACAATTCTTGTT
TT, Hairpin sequence for TRCN0000004896:
CCGGCCAGGATGAATGATGGTACTACTCGAGTAGTACCATCATTCATCCTGGTT
TT

Antibodies
Notch1 (sc-6014R, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, primary antibody dilution 1:200), Notch2 (#2420, Cell signaling Technology, Danvers, MA, primary antibody dilution 1:200), Notch3 (sc-5593, Santa Cruz Biotechnology, primary antibody dilution 1:200), Jagged2 (# 2205, Cell signaling Technology, primary antibody dilution 1:200), Jagged1 (sc-8303, Santa Cruz Biotechnology, primary antibody dilution 1:200), c-Myc (sc-40, Santa Cruz Biotechnology, primary antibody dilution 1:1000), Hes1 (ARP32372_T100, Avia Systems Biology, San Diego, CA, primary antibody dilution 1:200), Hey1 (ab22614, Abcam, Cambridge, MA, primary antibody dilution 1:400), cyclinD1 (sc-450, Santa Cruz Biotechnology, primary antibody dilution 1:200), Phospho-p44/42 MAPK (T202/Y204) (#4376, Cell signaling Technology), Phospho-AKT (S473) (#9271, Cell signaling Technology), anti-FLAG M2 monoclonal antibody (Sigma, primary antibody dilution 1:1000), β-actin (Sigma, primary antibody dilution 1:5000).

Western blot analysis
Fifty microgram of proteins were separated by electrophoresis in 6–12% SDS-polyacrylamide gels, electro-transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with the indicated antibodies as recommended by the manufacturer. Antibody binding was detected by chemiluminescence (Perkin-Elmer, Waltham, MA). Loading was normalized with β-actin (Sigma).

Cell line culture
Glioblastoma cell lines LN827, U87, deltaU87 (EGFR vIII over-expressing U87), U251 and LN428 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Mediatech, Manassas, VA), supplemented with 10% heat inactivated fetal bovine serum (Lonza, Basel, Switzerland), 1% penicillin-streptomycin (Mediatech, Manassas, VA) and 2% L-glutamine (Mediatech, Manassas, VA). 293FT cells were cultured in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were incubated at 37°C, 5% CO₂.

Neurosphere cultures
The human GBM stem cell line BT37 was derived from a patient who underwent surgery at Brigham and Women’s Hospital patients according to approved protocols. The BT70 and
BT74 lines were obtained from GBM xenografts received from C. David James (University of California, San Francisco, CA). These xenografts were derived from glioblastoma biopsies and implanted into immunocompromised mice and passaged in mice. Dissected xenografts were washed in artificial cerebrospinal fluid (CSF) and manually dissociated into single cells. Red blood cells were removed using Lympholyte-M (Cedarlane, Burlington, NC). The cells were cultured in DMEM/F12 (with L-glutamine, Invitrogen) medium containing glucose (0.3%), penicillin/streptomycin (50 μg/ml), Apo-transferrin (0.1mg/ml), Progesterone (20nM), Sodium selenite (30nM), putrescine (60μM), insulin (25μM/ml), sodium bicarbonate (3mM), HEPES (10mM), 20ng/ml EGF, 10ng/ml LIF, and 20ng/ml FGF. Live cells were counted using a hemocytometer and trypan blue exclusion (30,48).

Secondary neurosphere assay

Neurospheres are mechanically dissociated into single cells and viable cells counted using trypan blue exclusion. Approximately 20,000–100,000 viable cells were used per treatment condition (done in triplicates) and the cells were allowed to form spheres for five days after which the numbers of neurospheres were counted by microscopy (Figure 6B (b)).

Compound Treatment

For inhibitor treatments, cells were grown in their normal medium and treated with 10 μM, 20 μM of GSI MRK-003 (Merck Research Laboratories, Boston, MA) or DMSO (Sigma) for 24 or 48 hours as indicated in the text.

Lentiviral transduction

To produce lentivirus, 293FT cells were plated at 3x10^6 in 100mm dishes. DN-MAML1, PCSCGW2-IRE-RES-GFP lentiviral vectors, Lentiviral vectors targeting Notch1, Notch2 or PKL1.1 scrambl control plasmid together with packing plasmid pCMV_dr8_91 and pseudotyped envelope pMD2-VSV-G were transfected into the cells at ratio 1:1:1 using Fugene 6 reagent (Roche). Virus was collected 48 and 72 hours post-transfection. Target cells were infected twice with 0.8ml virus plus 0.2ml fresh complete medium with 8μg/ml Polybrene (Sigma) for 6 hours and replaced with fresh medium (49). For neurosphere infection, 110 ml of virus was concentrated by ultracentrifugation using SW-28 rotor and rotated at 19500 rpm at 4°C for 3 hours. The pellet was re-suspended in 360 μl serum free DMEM over-night. Fifty micro-liter of virus was used to infect 100,000 viable cells.

Quantitative real-time reverse transcription PCR (qPCR)

Total RNA was isolated by the Trizol method (Invitrogen) and cDNA was generated from 800ng of total RNA (Taqman Reverse Transcription Kit, Applied Biosystem, Foster City, CA). qPCR was performed from the cDNA samples using ABI PRISM 7500 sequence detector (Applied Biosystems, Foster City, CA) and the PCR products were measured by SYBR green method (Applied Biosystems, Foster City, CA). All samples were amplified in triplicates. The relative change of transcript amount in each sample was determined by normalizing with the GAPDH mRNA expression levels. The relative RNA expression of genes was also normalized with GAPDH amount (50). Primer sequences are listed in Supplementary table 4.

Cell growth, MTS assays, cell cycle analysis and apoptosis assay

For cell growth assay, 2x10^5/2ml GBM cells were plated in 6 well plates, infected with lentivirus plus fresh medium twice for six hours. On day 5 post infection, cells were trypsinized and resuspended in 500μl medium, 25μl of cells were counted using a hemocytometer by trypan blue exclusion. Rest of the cells were diluted to 4 ml, 2 ml of cells (1:2 dilution) were plated back to 6 well plates and count on day8 or day9.
For MTS proliferation assay (Promega, Madison, WI), cells were plated at 1–2×10^3/50μL in 96-well plate. 50μL growth medium diluted with MRK-003 or DMSO were added three hours later to make the final concentration as indicated in the text. MTS activities were measured on day 5 at OD 490 using the Elisa micro-plate reader (Spectra Max190).

For cell cycle assay, 2×10^5/2ml GBM cells were plated in 6 well plates, infected with lentivirus plus fresh medium twice for six hours. On day 4 post infection, cells were trypsinized, washed once with ice cold 1×PBS (Mediatech, Manassas, VA), fixed with 40% ethanol for 30 mins, treated with 500μL 500μg/ml RNase A (Sigma) for 30mins at 37°C and stained with 0.0046mg/ml propidium iodide (Sigma) for 30 mins in dark at room temperature. DNA amount was measured by flow cytometry analysis (BD FACScans). Cell cycle distribution was determined by ModFit software (Verity software House, Topsham, ME). A minimum of 10,000 events was collected to perform each experiment.

For apoptosis assay, 1×10^5/2ml GBM cells were plated in 6 well plates. 2 ml growth medium diluted with 20 μM MRK-003 or DMSO were added the second day or infected with DN-MAML1 or GFP lentivirus twice for 6 hours. Cell viability was measured after 24 or 48 hours of treatment using the Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Indianapolis, IN). Briefly, cells were trypsinized and washed once with 1×PBS. Cell pellet was resuspended in 100μl buffer 3 together with 2μl propidium iodide (PI) and 2μl annexin-V, incubated for 15 mins in dark and subjected to flow cytometry analysis (BD FACScans).

**In vivo murine xenograft model**

For LN827, cells were infected twice with DN-MAML1 or GFP control virus. On day three post transduction, 1×10^6 cells were injected intracranially into SCID (NOD/SCID mice from Taconic) mice using stereotactic apparatus (48). For BT37 studies, tumors grown in flanks of SCID mice were dissected out, dissociated, plated and immediately treated in vitro for 24 hours with either DMSO or MRK-003. Cells were then manually dissociated and counted. Cells were resuspended in PBS at concentration of 2×10^4 viable cells/5 μl, and implanted into right striatum as previously described (48). Mice were sacrificed when morbid and the date was recorded for survival analysis. Brain tumors form both DN-MAML1 or control mice were excised, snap frozen in liquid nitrogen and stored at −80°C.

**Statistical Analysis**

The values were shown as the mean ± SD. Comparison were performed using student’s t test (GraphPad Software, Inc, San Diego, CA). Significant p values were shown as p<0.05 (*), p<0.01(**), p<0.001(***). For survival analysis, Logrank Test was performed using Prism 4 software (GraphPad Software, Inc, San Diego, CA, 2005).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**


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Figure 1. Expression of Notch signaling components in GBM cells

A. qPCR analysis of Notch receptors expression in GBM cells. Results were generated from two or three independent passages of GBM cells, and each bar represents one independent preparation of an RNA sample.

B. Western blot analysis of protein levels of Notch signaling components in GBM cell lines. * indicates non-specific band.
Figure 2. A dominant negative form of mastermind-like 1 (DN-MAML1) inhibited Notch signaling in GBM cells and prevented GBM cell growth in vitro and in vivo

A. qPCR analysis showed DN-MAML1 transduction inhibited the expression of Notch target genes, C-MYC, HEY1 and HES1 in GBM cells as compared to GFP. The total RNA was collected 48 hours post-transduction and the experiments were performed in triplicate (n=3). The p values between the DN-MAML1 transduced cells and controls were indicated as followed: * p<0.05, ** p<0.01, and *** p<0.001.

B. DN-MAML1 inhibited GBM cell growth in vitro. Cells were infected twice with DN-MAML1 or GFP control viruses and the infected cells were counted by trypan blue exclusion on day 5 post-transduction. Cell growth of GFP transduced cells were set to 100%. * p<0.05, ** p<0.01, *** p<0.001 compared to GFP control virus infected GBM cells, n≥3.

C (a). DN-MAML1 inhibited GBM cell growth in vivo. Kaplan-Meier survival curves of mice harboring intracranial LN827 GBM cells transduced with DN-MAML1 or GFP control virus (p=0.0006).
C (b). qPCR analysis showed that DN-MAML1 inhibited the expression levels of Notch target genes, *C-MYC, HES1* and *HES5* in LN827 GBM xenografts compared to GFP control. Brain tumors were excised at the sacrifice time, snap frozen by liquid Nitrogen. Total RNA of the brain tumor formed from DN-MAML1 or GFP control virus treated LN827 cells were extracted and subjected to qPCR analysis. **p<0.01, ***p<0.001 n=2
Figure 3. DN-MAML1 expression caused G1 arrest, down-regulated CYCLIN D1 expression and induced apoptosis in GBM cells

A. Cell cycle distribution of GBM cells on day 4 post-transduction with DN-MAML1 lentivirus or GFP control virus. Cell cycle was determined by propidium iodine staining followed by FACS analysis, n≥3.

B. DN-MAML1 induced apoptosis in GBM cells. LN827, deltaU87 and U87 cells were infected twice with lenti-virus harboring DN-MAML1 or GFP. Forty-eight hours after transduction, apoptosis assay was performed by PI and Annexin-V staining and analyzed by flow cytometry, *p<0.05, **p<0.01, ***p<0.001 compare the % of apoptosis cells from DN-MAL1 expressing to GFP expresing cells, n=3.

C. qPCR analysis showed DN-MAML1 inhibited CYCLIN D1 expression in LN827, deltaU87 and LN428 cells. Total RNA was collected from cells 48 hours post-transduction with DN-MAML1 lenti-virus or GFP control virus. CYCLIN D1 expression of GFP transduced cells were set to 100%. *p<0.05, **p<0.01, ***p<0.001 compared to GFP control virus infected cells, n=3.
Figure 4. GSI MRK-003 treatment down-regulated Notch target gene expression, inhibited GBM cell growth and induced apoptosis

A. qPCR analysis showed that GSI MRK-003 inhibited C-MYC and HES1 expression. Total RNA of U87 and deltaU87 was collected 48 hours after indicated treatment. RNA expression of control (DMSO) treated cells were set to 100%, n=3.

B. qPCR analysis showed that GSI MRK-003 inhibited CYCLIN D1 expression. Total RNA from LN827, deltaU87 and LN428 was collected 48 hours after indicated treatment. RNA expression of control (DMSO) treated cells were set to 100%, n=3.

C. GSI differentially inhibited GBM cell line growth. MTS assay were performed on day 5 of treatment, n=3.

D. MRK-003 induced apoptosis in GBM cells. Twenty four and 48 hours after 20μM of MRK-003 treatment, apoptosis assay was performed by PI and Annexin-V staining and analyzed by flow cytometry. *p<0.05, **p<0.01, ***p<0.001 compare the % of apoptosis cells from MRK-003 treated to DMSO treated cells, n=3.
Figure 5. Notch1 and Notch2 knockdown differentially inhibited GBM cell growth

A. Western blot analysis showed down-regulation of Notch1 and Notch2 protein levels. LN827 cell lysate were prepared on day 5 post-transduction and subjected to western blot analysis. The same membrane was stripped and probed with Notch1, Notch2, Notch3 and β-actin antibodies to show the specificity of knocking-down, and loading control. *Indicates non-specific band. A representative of three independent experiments.

B. qPCR analysis showed knocking-down Notch1 or Notch2 inhibited HEY1 expression in deltaU87 and U251 cells compared to scramble control. Total RNA was collected from cells 48 hours post-transduction with lenti-virus harboring shRNAs targeting Notch1, Notch2 or scramble control. HEY1 expression of scramble control transduced cells was set to 100%. *p<0.05, **p<0.01, ***p<0.001 compared to control, n=3.

C. Knockdown of Notch1 or Notch2 differentially inhibited GBM cell growth. Cells were transduced twice with viruses harboring shRNAs targeting (a) Notch1 (#a and #b), or (b) targeting Notch2 (#a and #b) or scramble control shRNA, counted by trypan blue exclusion on day 5 post-transduction. Cell growth of scramble control virus infected cells was set to
100%. *p<0.05, **p<0.01, ***p<0.001 compared to scramble control virus treated cells, n≥3
Figure 6. Inhibition of Notch signaling prevented human GBM-derived neurosphere growth in vitro and in vivo

A. DN-MAML1 inhibited human GBM-derived neurosphere (BT70, BT74 and BT37) sphere formation in vitro. Neurospheres were transduced by either DN-MAML1 or GFP virus and sphere numbers were counted on day 10 post transduction. *p<0.05 compared to GFP control virus transduced stem cells, n=3.

B. GSI MRK-003 inhibited BT37 GBM neurosphere formation.
(a) GSI inhibited BT37 GBM neurosphere formation in a dose-dependent manner. Neurosphere numbers were counted on day 5 upon treatment. DMSO control treated BT37 sphere number was set to 1.0 (A representative of a triplicate experiment, n=3).
(b) A representative picture of GSI treatment inhibited BT37 neurosphere formation in a dose-dependent manner.

C. Pretreatment with GSI inhibited BT37 GBM neurosphere growth in vivo. Kaplan-Meier survival curve of mice harboring intracranial BT37 GBM neurosphere pretreated for 24 hours with 10μM of MRK-003 or DMSO control (p=0.0348).