Published in final edited form as:

Cancer Res. 2014 August 15; 74(16): 4536–4548. doi:10.1158/0008-5472.CAN-13-3703.

Human *Brat* ortholog *TRIM3* is a tumor suppressor that regulates asymmetric cell division in glioblastoma

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Abstract

Cancer stem cells, capable of self-renewal and multipotent differentiation, influence tumor behavior through a complex balance of symmetric and asymmetric cell divisions. Mechanisms regulating the dynamics of stem cells and their progeny in human cancer are poorly understood. In *Drosophila*, mutation of *brain tumor (brat)* leads to loss of normal asymmetric cell division by developing neural cells and results in a massively enlarged brain composed of neuroblasts with neoplastic properties. Brat promotes asymmetric cell division and directs neural differentiation at least partially through its suppression on Myc. We identified *TRIM3* (11p15.5) as a human ortholog of *Drosophila brat* and demonstrate its regulation of asymmetric cell division and stem cell properties of glioblastoma (GBM), a highly malignant human brain tumor. *TRIM3* gene expression is markedly reduced in human GBM samples, neurosphere cultures and cell lines and its reconstitution impairs growth properties *in vitro* and *in vivo*. *TRIM3* expression attenuates stem-like qualities of primary GBM cultures, including neurosphere formation and the expression of stem cell markers CD133, Nestin and Nanog. In GBM stem cells, *TRIM3* expression leads to a greater percentage dividing asymmetrically rather than symmetrically. As with Brat in *Drosophila*, *TRIM3* suppresses *c-Myc* expression and activity in human glioma cell lines. We also demonstrate a strong regulation of Musashi-Notch signaling by *TRIM3* in GBM neurospheres and neural stem cells that may better explain its effect on stem cell dynamics. We conclude that *TRIM3* acts as a tumor suppressor in GBM by restoring asymmetric cell division.

Keywords

Glioblastoma; Asymmetric; Stem cell; Brat; *TRIM3*

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Conflict of Interest Statement: The authors have no conflicts of interest to disclose.

Introduction

Glioblastoma (GBM) is the most malignant primary brain tumor in adults[1]. Glioma stem cells (GSCs), or brain tumor initiating cells, are a neoplastic subpopulation within GBMs that is highly tumorigenic and responsible for therapy resistance[2–4]. GSCs have characteristics similar to neural stem cells (NSCs), with the capacity for self-renewing cell division and multipotent differentiation along neural and glial lines [2, 5–7].

A shared trait and defining characteristic of stem cells is their ability to divide asymmetrically, giving rise to two non-identical daughter cells, with one maintaining stemness and the other developing a differentiated state[8]. Intrinsic molecular pathways that direct asymmetric cell division and stem-like behavior in the developing *Drosophila melanogaster* nervous system have been partially elucidated and may provide insight into similar regulatory networks in GSCs [9–12]. In *Drosophila* neural precursors, the asymmetric cellular localization of Numb, Prospero and Brain tumor (Brat) during cell division determines daughter cell fate. Daughter cells that inherit them progress to terminal differentiation, whereas daughter cells without them retain stem cell function and the ability to divide asymmetrically. Mutations in cell fate determinants (*numb*, *prospero*, *brat*) result in the inability of neural precursors to divide asymmetrically, leading to the accumulation of highly proliferative neuroblasts with pluripotent potential[11–15]. Brat and Prospero are sequestered into the daughter cell destined for differentiation, the ganglion mother cell (GMC), by the docking protein Miranda. Once segregated, Prospero acts as a transcriptional repressor that causes cell cycle exit, while Brat acts as a translational repressor that guides the cellular differentiation program, terminating asymmetric division and self-renewal [12, 14, 16].

Drosophila brat is characterized by a massively enlarged larval brain containing undifferentiated neuroblasts with neoplastic properties[10, 12, 13, 16]. Brat promotes differentiation at least partially through its translational repression of *Myc*[17, 18]. Based on its high degree of sequence homology and conserved functional domains, *TRIM3* has been identified as a human ortholog of *brat*. Its potential significance to gliomagenesis has been inferred from its deletion in 25–30% of GBM samples[19]. Recent studies of GBM by the Cancer Genome Atlas (TCGA) indicate that deletions involving 11p15, where *TRIM3* resides, are highly specific to the proneural and G-CIMP subclasses of GBM[20].

TRIM proteins belong to the family of E3 ubiquitin ligases that have a tripartite motif (TRIM) containing RING finger domain, one or two zinc-binding B-box domains and coiled-coil domains (Fig. 1A)[21]. TRIM proteins are known to regulate critical cellular processes including proliferation, apoptosis and transcriptional regulation. Their dysfunction has been implicated in developmental disorders and a variety of cancers. For example, TRIM13 and TRIM19 have tumor suppressive activity through a direct effect on the p53 regulatory protein, MDM2, while TRIM24 and TRIM28 suppress p53 stability and expression[21]. *TRIM* gene clusters are located on chromosomes 1, 4, 5, 6, 7, 11 and 17, and genes for more than 70 TRIM proteins that have been identified thus far[21]. TRIM3 was first identified and characterized as a brain-enriched RING finger protein (BERP) with its gene localized to chromosome 11p15.5 [22]. In this study, we provide functional evidence

that TRIM3 is a tumor suppressor in human GBM cell lines, patient-derived neurospheres and in *in vivo* xenografts. Mechanistically, TRIM3 reprograms glioma stem cells toward asymmetric cell division and differentiation through its regulation of c-Myc and Musashi-Notch pathways.

Materials and Methods

Cell culture

The human GBM cell lines U87MG, LN229, LN2308 and SF767, as well as their culture conditions, have been described previously [23, 24]. GBM neurosphere cultures were isolated from patient samples and established in culture as previously described and were utilized for experiments between passages 1 and 30 [25]. GBM neurospheres and normal human neural progenitor cells (NHNP; Lonza) were cultured in Neurobasal[®]-A media (Invitrogen) containing human epidermal growth factor (hEGF, Stemcell Technologies), basic fibroblast growth factor (bFGF, Stemcell Technologies) and GIBCO[®] B-27[®] supplement (Invitrogen) and N2 supplement (Invitrogen). Both GBM neurosphere and NHNP cultures show consistent expression of the stem cell markers Nestin and CD133. For GBM neurospheres, the percentage of Nestin⁺ cells depended on the cell lines and ranged from 10–18% and CD133⁺ ranged from 1–9%. For NHNP, the percentage of Nestin⁺ cells ranged from 3–20% and CD133⁺ ranged from 3–16%. Normal human astrocytes and human astrocytes sequentially transformed with hTERT, E6 and E7 have been previously described [26, 27].

Real time PCR

Total RNA was extracted using Trizol reagent (Invitrogen) and converted to cDNA using a cDNA synthesis kit (Applied Biosystems). Real time PCR assays were performed with the Power SYBR GREEN PCR master mix (Applied Biosystems) using a 7000 Sequence Detection System (Applied Biosystems). The sequences of human *TRIM3*, *p21*, *Cyclin D2* and *GAPDH* primers were designed as follows:

TRIM3 forward: 5'-GGCTGACTGGGGCAACAGCCGCATC-3',

reverse: 5'-ATCTGCAGAACCACTGTATGGTCCA-3';

p21 forward: 5'-ATTCAGCATTGTGGGAGAG-3' and

reverse: 5'-TGGACTGTTTTCTCTCGGCT-3';

Cyclin D2 forward: 5'-GCGGTGCTCCTCAATAG-3' and

reverse: 5'-TGGCATCCTCACAGGTC-3';

GAPDH forward: 5'-GAAGGTGAAGGTCGGAGTC-3' and

reverse: 5'-GAAGATGGTGATGG GATTTC-3'.

PCR was run at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 30 sec. Transcript levels from GBM samples and cells were normalized to *GAPDH* and reported as relative fold increase compared with normal brain tissues or normal human astrocytes by the 2^{-Ct} method as described [28].

TRIM3 over-expression constructs

Transient expression constructs: pRNAT-Trim3 includes a 3.2 kb full length *Trim3* cDNA; pLenti6-Trim3 includes only the coding region (open reading frame) and a V5 tag; and pFUW-Trim3 includes the open reading frame, but no tag.

Lentivirus constructs: FUW-Trim3-GFP and FUW-Trim3 expression constructs were used. The Trim3-GFP fusion cDNA construct was derived from pCMV6-AC-Trim3-GFP TrueORF clone (RG211739, Origene). The vector was partially digested with *FseI* to avoid the cutting the other *FseI* site inside the GFP coding region and with *BamHI*. The resulting 3 kb Trim3-GFP fusion cDNA fragment was ligated into the *BamHI* and *FseI* sites of the FUW vector, generating FUW-Trim3-GFP. The FUW-Trim3 construct was generated by amplifying the *Trim3* coding region and cloning into the *BamHI* and *HpaI* sites of the FUW vector. The following primers were used:

BamHI-TRIM3 5'-GGATCCGCCATGGCAAAGAGGGAGGACAGC-3' and

HpaI-TRIM3 5'-GTTAACCTACTGGAGGTAGCGATAGGCTTT-3'

The FUW-GFP expression vector was previously described [23].

TRIM3 short hairpin constructs

Three pairs of short hairpin oligos were designed against three different exon regions of the human *TRIM3* gene (NM_006458). Two oligos of each pair were annealed and ligated into the pRNATin H1.4 vector (GeneScript) which was linearized with *BamHI* and *XbaI* (NEB). The sequences of the oligos were as follows:

TRIM3-sh1-upper:

5'GATCCCATAGTCTGCCACAATTATGTCTTGATATCCGGACATAATTGTGGC
AGACTATTTTTTTTCCAAC3'and

TRIM3 sh1-lower:

5'TCGAGTTGGAAAAAATAGTCTGCCACAATTATGTCCGGATATCAAGACA
TAATTGTGGCAGACTATGG3'.

TRIM3-sh2-upper:

5'GATCCCATATGTGCCATTCTTGTGGTCTTGATATCCGGACCACAAGAATGG
CACATATTTTTTTTCCAAC-3'

and-lower:

5'TCGAGTTGGAAAAAATATGTGCCATTCTTGTGGTCCGGATATCAAGACC
ACAAGAATGGCACATATGG-3'.

TRIM3-sh3 upper:

5'GATCCCGTTGCAACACCTTCTGTTTGGCTTGATATCCGGCCAAACAGAAGG
TGTTGCAATTTTTTTTCCAAC-3' and

lower:

5'TCGAGTTGGAAAAAATTGCAACACCTTCTGTTTGGCCGGATATCAAGCCA
AAGAAGGTGTTGCAACGG-3'.

Lentiviral particle generation and infection

Lentiviral vectors (3 µg) were prepared by co-transfecting them with packaging plasmids (6 µg pCMV R8.72 and 1.5 µg pVSVG) in 2×10^5 293FT cells using Lipofectamine 2000 (Invitrogen). After overnight incubation, medium was replaced and 48h or 72h later the conditioned medium containing viral particles was collected, cleared of cell debris by centrifugation at 100g and stored at -80°C. To establish stable cell lines, 5000 cells each of U87MG or LN229 per well were seeded in 12 well plates. 1 ml of viral particles and 1 ml normal medium were added to each well and incubated for 48h. For GBM neurosphere, viral particles were collected and concentrated by ultracentrifugation at 25,000 rpm for 4h at 4°C. Viral pellets were resuspended in Neurobasal®-A media to prevent serum effects. Infection of cells and lentiviral-mediated gene expression were confirmed by demonstrating GFP expression by fluorescence microscopy and TRIM3 expression by western blot.

Cell proliferation assay

Stably infected cell lines U87MG-TRIM3, U87MG-GFP, LN229-TRIM3, and LN229-GFP cells were seeded in 96-well plates at a density of 2×10^3 cells per well in 100 µl of DMEM media with 10% FBS. Cell proliferation was analyzed at 0, 24, 48, 72, 96, and 168h by Sulforhodamine B assay (Sigma-Aldrich) [29].

Cell proliferation assay for neurospheres

Neurospheres from GBM samples and normal human neural progenitor cells (NHNP; Lonza) were measured with MTT based CellTiter96 Aqueous One Solution Cell Proliferation Assay Kit (Promega). 1000 cells in 100 µl medium were seeded per well in 96-well plate. 20 µl MTS solution added into each well and incubated at 37°C for 2 hours. Absorbance was read at 490 nm using a Synergy HT microplate reader (Biotek).

Colony formation assay

1×10^3 cells were resuspended in 0.5 ml 2X DMEM with 20% FBS, mixed with 0.5 ml 0.7% Ultrapure Agarose (Invitrogen) in water and the mixture was layered on top of the 6 well plates containing 2 ml of solidified 0.5% base agarose in 20% FBS 2X DMEM. 2 ml of the base agarose was loaded into each well of a 6 well plate and solidified the day before the experiment. After 5 weeks in culture, the colonies were stained with 0.5 ml 0.005% crystal violet (Sigma) in PBS and counted on a Gelcount Scanner (Oxford Optronix).

Flow cytometry

Analysis of stem cell markers in neurospheres was performed using antibodies against CD133 (Miltenyi Biotech) and Nestin (Abcam) Analysis was done using BD FACSCanto II (BD).

PKH26 assay

1×10^6 GBM neurosphere cells were dissociated with Accutase (Innovative Cell Technology) followed by staining with PKH26 Red fluorescent Cell Linker Mini Kit (Sigma-Aldrich). Briefly, dissociated cells were washed with 5 ml PBS at 400g and resuspended in 0.5 ml Diluent C. 4×10^{-6} molar PKH26 dye was added to cells. After staining for 5 min, the reaction was stopped by adding 1 ml PBS with 1% BSA followed by 2 ml Neurobasal-A[®] media. Cells were mixed by inverting tubes several times and collected by centrifuging at 400g. After washing 3 times with Neurobasal-A[®] media, stained neurosphere cells were cultured for 2 weeks for secondary neurosphere formation. Secondary neurospheres were then dissociated with Accutase and cells were sorted on a BD FACS Aria II Cell Sorter (BD). Sorted neurosphere cells were divided into three groups based on PKH26 staining intensity: PKH^{high}, PKH^{low}, and PKH^{negative} cells. 50 cells from each group were seeded onto each well of a 96-well plate and incubated under normal conditions. Symmetric and asymmetric cell divisions were monitored and photographed using a Zeiss Ax-10 fluorescence microscope (Zeiss).

Dual-luciferase reporter assay

A Signal Reporter Assay Kit (SA Biosciences) was used to measure c-Myc promoter driven luciferase expression.

Western blot

Western blot was performed as previously described[23]. Antibodies used were against c-Myc (BD Pharmingen), TRIM3 (GeneTex), β -actin, serving as a loading control (Santa Cruz Biotechnology and GeneTex), p21^{cip}, p27^{kip1}, Cyclin D2, Notch, Numb, Hes1, and MS1 (Musashi)(all Cell Signaling). Goat-anti-mouse IgG or goat-anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) were used as secondary antibodies. Enhanced chemiluminescence (ECL) was used for detection (Thermo Scientific).

Immunofluorescence analysis

Immunofluorescent staining was performed using antibodies against Nanog (1:100 BD Bioscience), Nestin (1:20, Abcam), c-Myc (1:500 Cell Signaling), CD 133 (1:20 Miltenyi Biotech). For intracellular staining, cells were fixed and permeabilized with BD Cytofix/Cytoperm/Permeabilization kit (BD Biosciences). Images were captured via Zeiss Axio Observer microscope. (Carl Zeiss Microimaging, Inc).

DNA copy number and somatic mutation data

We downloaded copy number and somatic mutation data of GBM samples collected in The Cancer Genome Atlas dataset from cBioPortal for Cancer Genomics [30]. The Genomic Identification of Significant Targets in Cancer (GISTIC) 2.0 was applied to DNA copy-number data to find driver genes targeted by somatic copy-number alterations accountable for cancer progression [31, 32]. We determined hemizygous and homozygous loss *TRIM2*, *TRIM3*, *TRIM32*, and *TRIM71* for 497 patients. Somatic mutation and copy number data were acquired for *CDKN2A*, *EGFR*, *IDH1*, *NF1*, *PDGFRA*, *TP53*, *PTEN*, *RBI* [33].

Gene expression data

Normalized gene expression data from the Agilent microarray platform was obtained through cBioPortal. We correlated in pairs the gene expression of *TRIM2*, *TRIM3*, *TRIM32*, and *TRIM71* with that of *MYC*, *NOTCH*, *NUMB*, *MSI*, and *NANOG* with Pearson's correlation, respectively. Correlation coefficients and p-values were computed with gene expression data of 547 patients.

Correlates of mRNA data with survival

Clinical information including patient age, gender, treatment history, survival, and censoring status was downloaded from the TCGA portal [34]. Survival differences were investigated between groups of patients with the upper and lower quartile of *TRIM3* expression. A Kaplan-Meier plot was produced and log-rank testing was carried out with survival data.

Transcriptional subtype class

TCGA GBMs are subdivided into four transcriptional subtypes based on unsupervised hierarchical clustering (Proneural (PN), Neural (NR), Classical (CL) and Mesenchymal (MS))[35]. Subtypes for samples absent from this data set were determined with Prediction Analysis of Microarray (PAM) software version 2.21 using RMA normalized Affymetrix HT-HGU133 mRNA expression platform data. A sample expression average was computed for samples with multiple corresponding arrays. Unlogged expression was filtered to remove probes with a fold change less than 1.5 or an expression range less than 20.

Integration of *TRIM3* deletion with transcriptional class and genetic alterations

To investigate the relationship of *TRIM3* deletion with gene expression subtypes, we used the hypergeometric distribution to calculate the significance of *TRIM3* deletion as either enriched or depleted in a specific transcriptional subtype. We computed the p-value of the significance test as the sum of the probabilities of all cases as equal as or more extreme than the observed data. A test with p-value < 0.05 was considered significant. Similar tests were conducted to study the relationship between *TRIM3* deletion and GBM genetic alterations, including DNA copy number alterations and mutations of genes *CDKN2A*, *EGFR*, *IDH1*, *MDM2*, *NF1*, *NOTCH1*, *NOTCH2*, *PDGFRA*, *PTEN*, *P53*, *RB1*.

Animal studies

All animal experiments were performed under approved protocols of the institutional animal use and care committee. For intracranial tumor inoculation, 1×10^5 cells were stereotactically implanted into the right striatum of 4–6 week old female athymic nude mice (12 mice/group) (*Foxn1nu*; Harlan). Animal survivals were compared using the Kaplan-Meier survival analysis and log-rank testing.

Statistical analysis

Quantitative data are expressed as mean $\pm$ SE. ANOVA was used to compare group differences. Statistical significance was assessed by Student's *t* test. Differences were considered to be significant when $p < 0.05$. Survival analysis was performed using Kaplan Meyer curves and significance was determined using the log-rank test.

Results

TRIM3 deletion and reduced expression in human gliomas

Potential human orthologs of *Drosophila brat*, based on sequence homology, include *TRIM2*[36], *TRIM3*[19, 22], *TRIM32*[37] and *TRIM71*[38], located on chromosomes 4q31.3, 11p15.5, 9q33.1 and 3p22.3, respectively. We focused our investigations on *TRIM3* as the human ortholog because: 1) *TRIM3* has the highest homology to *Drosophila brat*[10] (Fig. 1A); 2) *TRIM3* is specifically expressed in human brain, much like the expression of *brat* is specific to *Drosophila* neural tissue; 3) SNP-based analysis of *TRIM3* revealed loss in 25% of GBMs[19] and our own probing of The Cancer Genome Atlas (TCGA) data indicated that *TRIM3* has the highest frequency of deletion among TRIM family members. *TRIM3* was deleted in 22% of GBMs, whereas *TRIM2*, *TRIM32* and *TRIM71* were deleted in 12%, 11% and 10% of cases, respectively (Fig. 1B)[30].

Further *in-silico* analysis of *TRIM3* in human gliomas was performed using the independent REMBRANDT dataset, which includes all histologies and grades of gliomas, including astrocytomas and oligodendrogliomas of grades II, III and IV.[39] *TRIM3* (11p15.5) deletions were noted in 24% of all glioma samples (Fig. 1C) and were seen in both astrocytic and oligodendroglial histologies, grades II, II and IV. Deletions were as frequent in the lower grade neoplasms as GBM, grade IV, suggesting that *TRIM3* loss is an early neoplastic step.

Interestingly, we found that those GBMs with *TRIM3* deletion were highly enriched in the proneural transcriptional class, as defined by the TCGA, ($p = 1.23 \times 10^{-7}$) and depleted in the classical transcriptional class ($p = 8.71 \times 10^{-11}$)[30, 35]. In addition, *TRIM3* deletion was found to be highly enriched in *IDH1* mutant ($p=0.00031$), *PDGFRA* amplified ($p=0.00061$) and *TP53* mutant ($p=0.00013$) GBMs, all of which are characteristic of the proneural transcriptional class. The association of *IDH1* mutation with *TRIM3* deletion may partially explain the higher percentage of *TRIM3* deletions in grade II astrocytomas than grade III astrocytomas, since the latter have a lower frequency of IDH mutations[40]. *TRIM3* deletion was depleted in *EGFR* amplified ($p=8.4459 \times 10^{-8}$), *CDKN2A* deleted ($p=1.71128 \times 10^{-5}$) and *PTEN* deleted ($p=0.0056$) GBMs, which are all typical of the classical expression class. The strong association of *TRIM3* deletion with the proneural transcriptional class suggests that its loss is associated with gene expression related to neural developmental and proliferation, since these categories are enriched in this expression class[35].

TRIM3 gene expression was substantially lower in GBMs and lower grade gliomas (astrocytomas, oligodendrogliomas of grades II and III) as compared to normal brain (data not shown)[41]. In TCGA data, we found that *TRIM3* deletion in GBM (22%) was associated with reduced gene expression as compared to non-deleted GBMs ($p = 0.000097$). Since *TRIM3* expression was also noted to be low in GBMs without *TRIM3* deletion, we explored other mechanisms that might explain low gene expression. We compared DNA methylation levels of *TRIM3* to that of *glial fibrillary acidic protein* (*GFAP*) and found that *TRIM3* was methylated to a greater degree than *GFAP* ($n=112$; $p = 0.0041$), suggesting the DNA methylation might explain reduced expression [30]. While *TRIM3* deletion was not associated with a shorter survival among GBMs, we found that those GBMs with the highest

gene expression (top quartile) had a longer survival than those with lowest gene expression, yet this did not reach statistical significance ($p = 0.28$) (Fig. 2A)[30]. This slightly shorter survival associated with low *TRIM3* expression may be due to the association of *TRIM3* loss with the proneural transcriptional class, which has the shortest survival among GBM classes once the *IDH* mutant tumors have been removed from this group.[20] While *TRIM3* loss was also associated with *IDH1* mutations, which have prolonged survivals, there were very few *IDH1* mutants in our study TCGA set ($n = 8$ *IDH1* mutants; $n = 85$ proneural GBMs).

To verify *in silico* findings, we analyzed *TRIM3* transcript levels in human GBM tissue samples and cell lines with quantitative real-time PCR and found that *TRIM3* mRNA expression was substantially lower in GBMs than normal human brain (Fig. 2B). Similarly, normal human astrocytes in culture showed higher basal expression of *TRIM3* transcript than GBM cell lines or human astrocytes that were sequentially transformed with hTERT, E6 and E7 oncoproteins (Fig. 2C). *TRIM3* protein expression could be consistently identified as an 80 kDa band in normal brain (lanes 1–3) but was markedly reduced in human GBM samples (lanes 4–9) (Fig. 2D). The samples shown in Fig. 2D do not correspond to those samples in Fig. 2B. As compared to control tissues, *TRIM3* protein levels were reduced in GBM samples to a greater extent than mRNA levels (Fig. 2B). Furthermore, *TRIM3* was not detectable by Western blot in any of the GBM cell lines (data not shown).

***TRIM3* Expression Suppresses Glioma Proliferation and Colony Formation**

To better understand the functional consequences of *TRIM3* loss in glioma progression, we focused on lentiviral-mediated restoration of *TRIM3* protein expression in GBM cell lines and neurospheres. Restoration of *TRIM3* protein in GBM cell lines (Fig. 3A) and neurospheres (Fig. 3B) led to a significant reduction in their *in vitro* growth over the course of 8 days, with the first signs noted around day 4. *TRIM3* over-expressing GBM cells also showed a marked reduction in anchorage-independent colony formation in soft agar over the course of 30 days, with only 12% of the colonies formed as compared to GFP-expressing controls (Fig. 3C). The differing band patterns for the actin loading controls on Western blots in Fig. 3 resulted from the use of two different antibodies. Combined, the results suggest that *TRIM3* restoration in GBM cells reduces their *in vitro* growth.

Conversely, to investigate effects of reducing *TRIM3* expression in normal brain cells, we used lentiviral-mediated knock down in normal human neural progenitor (NHNP) cells (Fig. 3D). NHNPs with *TRIM3* shRNA knockdown showed a significant increase in cell growth as compared to controls by day 3, indicating that *TRIM3* regulates the expansion of neural cells that are not transformed.

We next examined the effect of *TRIM3* expression on glioma growth *in vivo*. We intracranially injected 10^5 control or *TRIM3* expressing glioma cells into the parietal region of nude mice. Mice that received intracranial injections of U87 or U87-GFP control cells had 50% survivals at 33 and 38 days, respectively. Mice injected with U87-*TRIM3* had significantly longer 50% survival at 53 days (log-rank test, $p < 0.05$; Fig. 3E). Thus, *TRIM3* expression in GBM cell lines suppressed tumor growth both *in vitro* and *in vivo*.

TRIM3 Promotes Differentiation and Suppresses Stem Cell Markers in GBM Neurosphere Cultures

We next investigated the role of TRIM3 in regulating glioma stem cell properties using neurosphere cultures. We used flow cytometry to measure the expression of Nestin and CD133 in dispersed GBM neurosphere cells stably transfected with GFP or TRIM3-GFP constructs. The percentage of Nestin expressing cells was significantly higher in neurosphere cells expressing GFP (16%) than in those expressing TRIM3-GFP (3.5%) (Fig. 4A). The percentage of CD133+ cells was only modestly greater in neurosphere cells expressing GFP (0.71%) than those expressing TRIM3-GFP (0.63%). The percentage of neurosphere cells expressing both Nestin and CD133 in the GFP expressing cells, although small (0.46%), was slightly greater than that of expressing GFP-TRIM3 (0.26%).

To further investigate the effects of TRIM3 on stemness, we used immunofluorescence microscopy of whole GBM neurospheres and dispersed cells in culture expression under basal (no serum) (Fig. 4B). We found that TRIM3 expression was consistently associated with reduced expression of the stem cell marker Nanog under basal conditions (Fig. 4B). Thus, our data indicates that *TRIM3* expression may be capable of reprogramming GBM neurosphere cells from a stem cell phenotype towards a more differentiated phenotype.

TRIM3 Reduces the PKH-26-high Population in GBM Neurosphere Cultures

To interrogate the role of TRIM3 specifically in regulating cell division within the glioma stem cell compartment, we used PKH-26, a fluorescent membrane dye that is partitioned evenly to daughter cells at the time of cell division and is maintained in highest concentration by cells that divide slowly. Previous studies have shown that PKH-26-high cells demonstrate asymmetric cell division; have the greatest degree of stemness; and have the highest tumorigenic potential[42]. N08-74-GFP and N08-74-TRIM3 neurospheres were stained with PKH-26, allowed to divide for 14 days, and then subjected to flow cytometry. We found that there was a population shift with regard to PKH-26 expression, with N08-74-TRIM3 cells showing lower PKH-26 expression than N08-74-GFP. However, the percentage of PKH-26 high cells in N08-74-TRIM3 cells was lower (0.47%) than N08-74-GFP cells (1.27%), suggesting that TRIM3 attenuates this GSC population (Fig. 4C). Combined with the flow cytometry data on Nestin and CD133, our findings strongly suggest that the GSC population is substantially reduced in TRIM3 expressing neurospheres.

TRIM3 Reduces Sphere Formation and Favors Asymmetric Cell Division in GBM Stem Cells

We next investigated TRIM3 for its effect on the ability of primary GBM cultures to form neurospheres, a GSC-dependent function. Cultures established from GBM samples in neural basal media (lacking serum) follow a fairly consistent sequence, with dispersed single cells forming neurospheres ranging from 0.1 – 1 mm over the course of 96 hrs. We found that N08-74-TRIM3 cultures consistently formed fewer and smaller neurospheres than controls (N08-74 or N08-74-GFP cultures) (Fig. 5A). Thus, TRIM3 impairs the ability of GBM explants to form neurospheres.

We next evaluated the impact of TRIM3 expression on cell division, specifically within a PKH-26 high (GSC) population. After 14 days following PKH-26 staining, we isolated individual PKH-26-high cells from both the N08-74-GFP and N08-74-TRIM3-GFP cultures and re-plated them at low density (10 cells per well in 96 well plates). We observed and quantified cell division patterns in these two cell types. In N08-74-GFP PKH-high cells (Fig. 5B, *upper panel*), we found a consistent pattern of 1-2-4-8 cells (symmetric cell division, 76.2%; asymmetric cell division, 23.8%). Neurosphere cells that expressed TRIM3 (Fig. 5B, *lower panel*) showed multiple patterns of cell division, including 1-2-3-5, 1-2-3-6 and 1-2-3-4 (asymmetric cell division, 82.9%; symmetric cell division, 17.1%). Thus, within the PKH-high cell population, *TRIM3* expression leads to a switch from predominantly symmetric cell division to asymmetric cell division.

TRIM3 Represses c-Myc Expression and Activity in Human Gliomas and Astrocytes

Drosophila Brat protein promotes asymmetric cell division and neural differentiation at least partially through repression of c-Myc[12]. Based on this, we investigated the relationship between TRIM3 and c-Myc in human gliomas. In the TCGA GBM data, we observed that *c-Myc* was over-expressed and *TRIM3* was under-expressed in nearly all GBM samples (Fig. 6A). We also noted a statistically significant negative correlation between *TRIM3* expression and *c-Myc* in the same samples ($p < 0.05$, $\rho = -0.235$, Fig. 6B). To formally test the causative nature of this relationship, we stably transfected LN229 GBM cells with either GFP or TRIM3-GFP expressing lentiviral constructs and showed that TRIM3 was exclusive to the cytoplasm of TRIM3-GFP expressing cells, whereas no observable TRIM3 protein was detected in GFP control cells (Fig. 6C). As compared to control GFP expressing cells, nuclear c-Myc expression was significantly reduced in TRIM3-GFP cells (Fig. 6C). We further investigated the effects of TRIM3 on the expression of positively (Cyclin D2) and negatively (p21 and p27) regulated c-Myc target genes. With over-expression of TRIM3, Cyclin D2 levels were decreased, while p27 and p21 levels were markedly increased (Fig. 6D). We also examined effects of downregulating TRIM3 expression on p21 mRNA levels in normal human astrocytes, which have a high basal expression of TRIM3. Normal astrocytes were stably infected with *TRIM3*-shRNA constructs, resulting in significant knockdown of *TRIM3*, as well as a reduction in the level of p21 transcript levels (Fig. 6E). To confirm the suppressive effect of TRIM3 on c-Myc, we used a c-Myc driven luciferase reporter in U87MG and LN229 GBM cell lines infected with GFP or TRIM3-GFP expressing lentiviruses. In both cell lines, TRIM3 over-expression led to a significant reduction in luciferase activity (Fig. 6F). Collectively, these data confirm the suppressive effect of TRIM3 on c-Myc levels and transcriptional activity in cultured glioma cell lines.

TRIM3 Regulates the Musashi-Notch Pathway in Neural and GBM Neurospheres

To better understand mechanisms of TRIM3-mediated control of differentiation and cell division, we investigated the impact of TRIM3 on c-Myc expression and its target genes in normal human neural progenitor (NHNP) cells and GBM neurosphere cultures, since these preparations contain a stem cell component. We established stable NHNP neurosphere lines using *TRIM3* knockdown (sh-Trim3) or GFP expressing (GFP) lentiviruses (Fig. 7A). NHNPs with *TRIM3*-shRNA showed significantly reduced TRIM3 protein and, as with glioma cell lines, showed increased levels of c-Myc expression compared to GFP controls.

Contrary to expectations and findings in established glioma cell lines (U87MG and LN229), expression of the c-Myc target gene p21 was not inhibited by increased c-Myc expression and the expression of Cyclin D2 was not altered. We further probed these cultures for signaling pathways regulated by TRIM3 that might explain the discrepancy between c-Myc expression and its target genes. Since the Notch pathway is known to regulate p21, we started by investigating the Musashi/Notch pathway. Musashi is an RNA binding protein that activates the Notch signaling by inhibiting Numb translation and preventing degradation of Notch-1 [43, 44]. Numb normally binds to the Notch-1 intracellular domain (NICD) and reduces the expression of downstream targets such as Hes-1. In NHNP control neurospheres (NHNP-GFP), we found that high basal TRIM3 expression correlated with low Musashi expression, high Numb expression and low Notch activity, as determined by Hes-1 expression. Inhibition of TRIM3 in these neurospheres (NHNP—sh-TRIM3) led to significant up-regulation of Musashi, down-regulation of Numb and increased expression of the Notch-1 target Hes-1 (Fig. 7B). Consistent with these findings in NHNP, restoration of TRIM3 in GBM neurospheres led to reduced levels of Musashi and Hes-1 (Fig. 7C, *left panel*). Immunofluorescence studies corroborated the inverse relationship between TRIM3 and Musashi expression (Fig. 7C, *right panel*). Thus, data from NHNP and GBM neurospheres strongly suggest a role of TRIM3 in regulating the Musashi-Numb-Notch signaling axis.

Discussion

The regulation of asymmetric stem cell division is complex and poorly understood in mammalian systems. Since the undifferentiated neuroblasts that accumulate in *Drosophila brat* have neoplastic properties, disruption of similar regulatory mechanisms in human brain tumors may impact stem cell dynamics and favor neoplastic growth [15]. Among human genes, we and others have found that *TRIM3* has the highest homology with *brat* [10, 19].

TRIM3 loss (11p15.5) is detected in ~25% of GBMs, as well as in lower grade gliomas. While gene expression of *TRIM3* was lowest in those GBMs with deletion, it was also reduced in nearly all GBMs based on analysis of TCGA data. Mechanisms responsible for low *TRIM3* expression in non-deleted gliomas are not known. However, it is possible DNA methylation may be at least partially responsible. The 11p15 region is a site of frequent dysregulated DNA methylation and loss of imprinting, with disrupted methylation in this region implicated in numerous developmental and neoplastic diseases [45, 46]. Our own analysis of TCGA GBMs indicated that *TRIM3* showed significantly increased methylation levels compared to *GFAP*, which may partially explain its reduced expression in non-deleted gliomas.

Interestingly, we found that *TRIM3* deletion was highly associated with the proneural transcriptional class of GBM, which is enriched for genes that regulate neural developmental and proliferation [35]. This tight association indicates that *TRIM3* loss is a marker for a specific transcriptional form of malignant glioma, potentially one with more neural stem-like qualities. In our own investigations of GBM patient samples, neurosphere cultures and established glioma cell lines, *TRIM3* mRNA and protein expression were low or absent in all samples. Our studies of the functional consequence of TRIM3 over-expression and

knockdown in GBM cell lines and neurospheres indicated that TRIM3 has tumor suppressive activity, decreases cellular proliferation *in vitro* and slows tumor growth *in vivo*. Since *Drosophila* Brat is a repressor of proto-oncogene c-Myc, we examined this relation in human gliomas and observed that TRIM3 indeed suppresses c-Myc expression and activation/repression of its target genes in established GBM cell lines [12, 15]. Others have also described the regulation of p21 by TRIM3 in glioma cell lines [47].

Since we were interested in furthering our investigations in cellular models that contain a stem cell population and mimic the human disease to a greater extent, we investigated neurosphere cultures derived from human GBM explants as well as their non-neoplastic counterpart, normal human neural progenitors (NHNP), which also grow as 3D neurospheres. In these preparations, we also observed that TRIM3 was capable of suppressing c-Myc; however, downstream target genes of c-Myc, such as p21 and Cyclin D2, did not correlate with c-Myc levels and suggested that other key regulatory networks that control proliferation and differentiation may also be under the control of TRIM3.

We observed that TRIM3 expression was strongly associated with suppressed expression of the stem cell markers Nestin, Nanog and Musashi, consistent with our hypothesis that it may antagonize stem-like behavior. Musashi (MSI) is an RNA-binding protein that promotes stem-cell self-renewal by activating Notch signaling. Musashi binds the 3' UTR of *Numb* mRNA and inhibits its translation. Numb normally binds to intracellular domain of Notch (NICD) to inhibit signaling. Thus, Musashi activates Notch signaling by removing the repression on NICD[48]. Musashi has been shown to promote growth and survival of glioma cells by activating Notch and PI3-K signaling[44]. Our results indicate that in NHNP and GBM neurospheres, TRIM3 regulates proliferation and differentiation through the TRIM3/ Numb/Notch pathway, in addition to c-Myc.

We observed that TRIM3 expression by glioma channels the cell population away from an undifferentiated, stem-like state. By flow cytometry, immunofluorescence and western blots, TRIM3 restoration in GBM neurospheres was associated with reduced expression of stem cell markers, such as Nestin, Musashi and Nanog. We also observed that TRIM3 expression within a stem cell population (PKH-high) leads to a greater proportion undergoing asymmetric cell division and reduces the ability of aggregated GBM cells to form neurospheres. Taken together, we conclude that loss of *TRIM3* during gliomagenesis increases the glioma stem cell population by disrupting asymmetric cell division and cellular differentiation.

Acknowledgments

This work was supported by US Public Health Service National Institutes of Health (NIH) grants R01CA149107(DJB), R01CA86335 (EGVM), the In Silico Center for Brain Tumor Research Contract ST12-1100 (NCI-SAIC Frederick); the Georgia Cancer Coalition (DJB); and the Winship Cancer Center Support Grant (P30 CA138292). We would like to thank Jennifer Shelton and Dianne Alexis at the Winship Cancer Tissue and Pathology shared resource for assistance in this work.

References

1. Ostrom QT, Gittleman H, Farah P, Ondracek A, Chen Y, Wolinsky Y, et al. CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006–2010. *Neuro Oncol.* 2013; 15(Suppl 2):ii1–ii56. [PubMed: 24137015]
2. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. *Nature.* 2004; 432:396–401. [PubMed: 15549107]
3. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature.* 2006; 444:756–760. [PubMed: 17051156]
4. Hadjipanayis CG, Van Meir EG. Brain cancer propagating cells: biology, genetics and targeted therapies. *Trends Mol Med.* 2009; 15:519–530. [PubMed: 19889578]
5. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell.* 2006; 9:157–173. [PubMed: 16530701]
6. Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. *Cell.* 2008; 132:645–660. [PubMed: 18295581]
7. Chen R, Nishimura MC, Bumbaca SM, Kharbanda S, Forrest WF, Kasman IM, et al. A hierarchy of self-renewing tumor-initiating cell types in glioblastoma. *Cancer Cell.* 2010; 17:362–375. [PubMed: 20385361]
8. Gonzalez C. Spindle orientation, asymmetric division and tumour suppression in *Drosophila* stem cells. *Nat Rev Genet.* 2007; 8:462–472. [PubMed: 17510666]
9. Gateff E. Malignant neoplasms of genetic origin in *Drosophila melanogaster*. *Science.* 1978; 200:1448–1459. [PubMed: 96525]
10. Arama E, Dickman D, Kimchie Z, Shearn A, Lev Z. Mutations in the beta-propeller domain of the *Drosophila* brain tumor (brat) protein induce neoplasm in the larval brain. *Oncogene.* 2000; 19:3706–3716. [PubMed: 10949924]
11. Caussinus E, Gonzalez C. Induction of tumor growth by altered stem-cell asymmetric division in *Drosophila melanogaster*. *Nat Genet.* 2005; 37:1125–1129. [PubMed: 16142234]
12. Betschinger J, Mechtler K, Knoblich JA. Asymmetric segregation of the tumor suppressor brat regulates self-renewal in *Drosophila* neural stem cells. *Cell.* 2006; 124:1241–1253. [PubMed: 16564014]
13. Bello B, Reichert H, Hirth F. The brain tumor gene negatively regulates neural progenitor cell proliferation in the larval central brain of *Drosophila*. *Development.* 2006; 133:2639–2648. [PubMed: 16774999]
14. Bowman SK, Rolland V, Betschinger J, Kinsey KA, Emery G, Knoblich JA. The tumor suppressors Brat and Numb regulate transit-amplifying neuroblast lineages in *Drosophila*. *Dev Cell.* 2008; 14:535–546. [PubMed: 18342578]
15. Knoblich JA. Asymmetric cell division: recent developments and their implications for tumour biology. *Nat Rev Mol Cell Biol.* 2010; 11:849–860. [PubMed: 21102610]
16. Lee CY, Wilkinson BD, Siegrist SE, Wharton RP, Doe CQ. Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. *Dev Cell.* 2006; 10:441–449. [PubMed: 16549393]
17. Sonoda J, Wharton RP. *Drosophila* Brain Tumor is a translational repressor. *Genes Dev.* 2001; 15:762–773. [PubMed: 11274060]
18. Harris RE, Pargett M, Sutcliffe C, Umulis D, Ashe HL. Brat promotes stem cell differentiation via control of a bistable switch that restricts BMP signaling. *Dev Cell.* 2011; 20:72–83. [PubMed: 21238926]
19. Boulay JL, Stiefel U, Taylor E, Dolder B, Merlo A, Hirth F. Loss of heterozygosity of TRIM3 in malignant gliomas. *BMC Cancer.* 2009; 9:71. [PubMed: 19250537]
20. Brennan CW, Verhaak RG, McKenna A, Campos B, Nounshmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. *Cell.* 2013; 155:462–477. [PubMed: 24120142]

21. Hatakeyama S. TRIM proteins and cancer. *Nat Rev Cancer*. 2011; 11:792–804. [PubMed: 21979307]
22. El-Husseini AE, Fretier P, Vincent SR. Cloning and characterization of a gene (RNF22) encoding a novel brain expressed ring finger protein (BERP) that maps to human chromosome 11p15.5. *Genomics*. 2001; 71:363–367. [PubMed: 11170753]
23. Rong Y, Belozarov VE, Tucker-Burden C, Chen G, Durden DL, Olson JJ, et al. Epidermal growth factor receptor and PTEN modulate tissue factor expression in glioblastoma through JunD/activator protein-1 transcriptional activity. *Cancer Res*. 2009; 69:2540–2549. [PubMed: 19276385]
24. Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, Diserens AC, et al. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. *Brain Pathol*. 1999; 9:469–479. [PubMed: 10416987]
25. Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell*. 2006; 9:391–403. [PubMed: 16697959]
26. Sonoda Y, Ozawa T, Hirose Y, Aldape KD, McMahon M, Berger MS, et al. Formation of intracranial tumors by genetically modified human astrocytes defines four pathways critical in the development of human anaplastic astrocytoma. *Cancer Res*. 2001; 61:4956–4960. [PubMed: 11431323]
27. Rong Y, Post DE, Pieper RO, Durden DL, Van Meir EG, Brat DJ. PTEN and hypoxia regulate tissue factor expression and plasma coagulation by glioblastoma. *Cancer Res*. 2005; 65:1406–1413. [PubMed: 15735028]
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-(Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
29. Yin S, Kaluz S, Devi NS, Jabbar AA, de Noronha RG, Mun J, et al. Arylsulfonamide KCN1 inhibits in vivo glioma growth and interferes with HIF signaling by disrupting HIF-1alpha interaction with cofactors p300/CBP. *Clin Cancer Res*. 2012; 18:6623–6633. [PubMed: 22923450]
30. <http://www.cbioportal.org/public-portal/> (last accessed February 14, 2014)
31. Beroukhi R, Getz G, Nghiemphu L, Barretina J, Hsueh T, Linhart D, et al. Assessing the significance of chromosomal aberrations in cancer: methodology and application to glioma. *Proc Natl Acad Sci U S A*. 2007; 104:20007–20012. [PubMed: 18077431]
32. Goringe KL, George J, Anglesio MS, Ramakrishna M, Etemadmoghadam D, Cowin P, et al. Copy number analysis identifies novel interactions between genomic loci in ovarian cancer. *PLoS One*. 2010; 5
33. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008; 455:1061–1068. [PubMed: 18772890]
34. <http://cancergenome.nih.gov/> (last accessed February 14, 2014).
35. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*. 2010; 17:98–110. [PubMed: 20129251]
36. Reymond A, Meroni G, Fantozzi A, Merla G, Cairo S, Luzi L, et al. The tripartite motif family identifies cell compartments. *EMBO J*. 2001; 20:2140–2151. [PubMed: 11331580]
37. Wulczyn FG, Cuevas E, Franzoni E, Rybak A. miRNAs Need a Trim : Regulation of miRNA Activity by Trim-NHL Proteins. *Adv Exp Med Biol*. 2011; 700:85–105. [PubMed: 21755476]
38. Loedige I, Gaidatzis D, Sack R, Meister G, Filipowicz W. The mammalian TRIM-NHL protein TRIM71/LIN-41 is a repressor of mRNA function. *Nucleic Acids Res*. 2013; 41:518–532. [PubMed: 23125361]
39. Cooper LA, Gutman DA, Long Q, Johnson BA, Cholleti SR, Kurc T, et al. The proneural molecular signature is enriched in oligodendrogliomas and predicts improved survival among diffuse gliomas. *PLoS ONE*. 2010; 5:e12548. [PubMed: 20838435]
40. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med*. 2009; 360:765–773. [PubMed: 19228619]
41. <https://caintegrator.nci.nih.gov/rembrandt/> (last accessed March 10, 2013).

42. Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, et al. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell*. 2009; 138:1083–1095. [PubMed: 19766563]
43. Okano H, Kawahara H, Toriya M, Nakao K, Shibata S, Imai T. Function of RNA-binding protein Musashi-1 in stem cells. *Exp Cell Res*. 2005; 306:349–356. [PubMed: 15925591]
44. Muto J, Imai T, Ogawa D, Nishimoto Y, Okada Y, Mabuchi Y, et al. RNA-binding protein Musashi1 modulates glioma cell growth through the post-transcriptional regulation of Notch and PI3 kinase/Akt signaling pathways. *PLoS One*. 2012; 7:e33431. [PubMed: 22428049]
45. Satoh Y, Nakadate H, Nakagawachi T, Higashimoto K, Joh K, Masaki Z, et al. Genetic and epigenetic alterations on the short arm of chromosome 11 are involved in a majority of sporadic Wilms' tumours. *Br J Cancer*. 2006; 95:541–547. [PubMed: 16909133]
46. Rumbajan JM, Maeda T, Souzaki R, Mitsui K, Higashimoto K, Nakabayashi K, et al. Comprehensive analyses of imprinted differentially methylated regions reveal epigenetic and genetic characteristics in hepatoblastoma. *BMC Cancer*. 2013; 13:608. [PubMed: 24373183]
47. Liu Y, Raheja R, Yeh N, Ciznadija D, Pedraza AM, Ozawa T, et al. TRIM3, a tumor suppressor linked to regulation of p21(Waf1/Cip1.) . *Oncogene*. 2014; 33:308–315. [PubMed: 23318451]
48. Nishimoto Y, Okano H. New insight into cancer therapeutics: induction of differentiation by regulating the Musashi/Numb/Notch pathway. *Cell Res*. 2010; 20:1083–1085. [PubMed: 20805843]

Figure 1. *TRIM3*, the human homolog of *Drosophila brat* is deleted in GBMs

A) Schematic of structure and homology of *Drosophila brat* gene with human genes- *TRIM2*, 3, 32 and 71. (Figure adapted with permission from Arama E et al[10]). **B)** Percentage of GBMs with deletion (hemizygous, homozygous and total deletion) of *TRIM2*, 3, 32 and 71 from data in the TCGA GBM dataset. **C)** Percentage of gliomas of differing histologies and grades with *TRIM3* deletion from data in the REMBRANDT data set (295 total gliomas).

Figure 2. *TRIM3* expression is reduced in human GBMs

A) Kaplan-Meier survival curve of patients in the TCGA GBM data set with highest quartile and lowest quartile expression of *TRIM3* (log-rank test, $p=0.28$). **B)** *TRIM3* mRNA expression GBM tissue samples, normal brain tissue and in **C)** human GBM cell lines as compared to normal astrocytes. Astrocytes transfected with E6, E7 and hTERT also show decreased *TRIM3* expression. **D)** Representative immunoblot showing *TRIM3* protein levels in normal brain (lanes 1–3) and GBM tissue samples (lanes 4–8). Actin served as the loading control.

Figure 3. TRIM3 expression reduces cell proliferation and glioma growth

A, B) Cell proliferation assay of **A)** U87 cells and **B)** N08-74 GBM neurospheres expressing GFP (controls) or TRIM3 **C)** Colony formation assay in LN229 GBMs expressing GFP (controls) or TRIM3 **D)** Cell proliferation assay of NHNP neurospheres expressing GFP or a *TRIM3* shRNA. Actin served as the loading control for A–D. **E)** Kaplan-Meier survival curve of mice intracranially injected with 10^5 U87MG glioma cells, including controls, GFP- or TRIM3-expressing (log rank test, $p < 0.05$).

Figure 4. TRIM3 regulates the stem cell population in GBM neurosphere cultures

A) Flow cytometry analysis of Nestin and CD133 expression in neurospheres N08-74-GFP (left) and N08-74-*Trim3* (right). *TRIM3* expression strongly represses Nestin expression and modestly suppresses CD133 expression (arrows) **B)** Immunofluorescence images from control (N08-74-GFP; left) and TRIM3 (N08-74-TRIM3 right) expressing GBM neurosphere cultures stained for Nanog (red) and DAPI (blue). **C)** Flow cytometry of PKH-26 stained GBM neurospheres N0874-GFP (left) and N08-74-*TRIM3* (right). *TRIM3* expression substantially reduced the percentage of PKH-26-high cells (arrows).

Figure 5. TRIM3 attenuates the stem cell population in GBM neurosphere cultures
A) Average size of neurospheres derived from GBM explant controls (N08-74, nontransfected), cultures transfected with GFP, or cultures transfected with TRIM3-GFP **B)** Phase-fluorescence time-lapsed microscopy images of first few divisions of PKH-26-high neurosphere cells expressing GFP (control, *upper panel*) or TRIM3 (*bottom panel*). Pie charts show percentage of asymmetric vs. symmetric cell divisions within each group.

Figure 6. TRIM3 suppresses c-Myc expression

A) mRNA expression levels of *c-Myc* and *TRIM3* in the TCGA GBM dataset. **B)** Correlation of *c-Myc* and *TRIM3* mRNA expression in the TCGA GBM data set (*Trim3* Vs. *c-Myc*: $p < 0.05$, $r_{\text{low}} = -0.317$, $r_{\text{high}} = -0.151$). Representative immunoblots showing decreased c-Myc protein expression in **C)** LN229 cells stably transfected with *TRIM3* expressing constructs show lack of c-Myc protein expression as compared to the controls. **D)** Western blots of downstream effectors of c-Myc (p21, p27 and cyclin D2) in LN229 gliomas stably transfected with either GFP or *TRIM3*-GFP. Actin served as the loading control for C and D. **E)** RT-PCR analysis showing strong correlation between *TRIM3* and *p21* mRNA levels in normal astrocytes stably transfected with *Trim3* shRNA or vector controls **G)** *c-Myc* promoter driven Luciferase assay in U87 and LN229 GBM cell lines transfected with either GFP or *TRIM3*-GFP showing reduced c-Myc activity in *TRIM3* expressing glioma cell lines

Figure 7. TRIM3 regulates Musashi/Numb signaling

A,B) Immunoblots of lysates from NHNP neurospheres with TRIM3 shRNA knockdown show **A)** modest upregulation of c-Myc but no corresponding effect on the levels of c-Myc targets (p21 and Cyclin D2), **B)** increased Musashi and Notch-1 target expression (Hes-1)

C) Immunoblot (*left panel*) from control (GFP) and TRIM3 (Trim3-GFP) expressing GBM neurospheres (N08-74) shows decreased Musashi, Numb and Notch-1 downstream effector Hes-1. Actin served as the loading control for **B** and **C**. **D)** Immunofluorescence images

from a GBM neurosphere (N08-74) transfected with GFP (control) or TRIM3 (Trim3-GFP) showing decreased staining for Musashi (red) in TRIM3 expressing cells.