

# Nonreceptor Tyrosine Kinase BMX Maintains Self-Renewal and Tumorigenic Potential of Glioblastoma Stem Cells by Activating STAT3

Olga A. Guryanova,<sup>1</sup> Qiulian Wu,<sup>1</sup> Lin Cheng,<sup>1,3</sup> Justin D. Lathia,<sup>1</sup> Zhi Huang,<sup>1</sup> Jinbo Yang,<sup>2</sup> Jennifer MacSwords,<sup>1</sup> Christine E. Eyler,<sup>1,5</sup> Roger E. McLendon,<sup>6</sup> John M. Heddleston,<sup>1</sup> Weinian Shou,<sup>4</sup> Dolores Hambardzumyan,<sup>1</sup> Jeongwu Lee,<sup>1</sup> Anita B. Hjelmeland,<sup>1</sup> Andrew E. Sloan,<sup>7</sup> Markus Bredel,<sup>8</sup> George R. Stark,<sup>2</sup> Jeremy N. Rich,<sup>1,\*</sup> and Shideng Bao<sup>1,\*</sup>

<sup>1</sup>Department of Stem Cell Biology and Regenerative Medicine

<sup>2</sup>Department of Molecular Genetics

Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195, USA

<sup>3</sup>Experimental Center, The First People's Hospital, Shanghai Jiaotong University, Shanghai, 200080, China

<sup>4</sup>Department of Pediatrics, Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202, USA

<sup>5</sup>Department of Pharmacology and Cancer Biology

<sup>6</sup>Department of Pathology

Duke University Medical Center, Durham, NC 27710, USA

<sup>7</sup>Brain Tumor and Neuro-Oncology Center, University Hospitals, Case Western Reserve University, Cleveland, OH 44106, USA

<sup>8</sup>Departments of Radiation Oncology, Genetics, and Cell Biology, University of Alabama at Birmingham School of Medicine, Birmingham, AL 35249, USA

\*Correspondence: richj@ccf.org (J.N.R.), baos@ccf.org (S.B.)

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## SUMMARY

Glioblastomas display cellular hierarchies containing tumor-propagating glioblastoma stem cells (GSCs). STAT3 is a critical signaling node in GSC maintenance but molecular mechanisms underlying STAT3 activation in GSCs are poorly defined. Here we demonstrate that the bone marrow X-linked (BMX) nonreceptor tyrosine kinase activates STAT3 signaling to maintain self-renewal and tumorigenic potential of GSCs. BMX is differentially expressed in GSCs relative to nonstem cancer cells and neural progenitors. BMX knock-down potently inhibited STAT3 activation, expression of GSC transcription factors, and growth of GSC-derived intracranial tumors. Constitutively active STAT3 rescued the effects of BMX downregulation, supporting that BMX signals through STAT3 in GSCs. These data demonstrate that BMX represents a GSC therapeutic target and reinforces the importance of STAT3 signaling in stem-like cancer phenotypes.

## INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and lethal type of primary brain tumor with a median survival of 12–15 months despite optimal therapy (Furnari et al., 2007; Wen and Kesari, 2008). GBM displays striking cellular heterogeneity and hierarchy in differentiation status. The fraction of

cancer cells at the apex of the hierarchy are called glioblastoma stem cells (GSCs), functionally defined by extensive self-renewal, multilineage differentiation potential and propagation of tumors that recapitulate the tissue architecture and cellular hierarchy of the parental lesion (Park and Rich, 2009; Rosen and Jordan, 2009; Vescovi et al., 2006; Zhou et al., 2009). We and others have demonstrated that GSCs interact with

### Significance

Glioblastomas are highly lethal brain tumors containing self-renewing cancer stem cells that promote tumor angiogenesis, invasion, and therapeutic resistance—each contributing to treatment failure. The elucidation of molecular mechanisms that specifically control the stem cell-like properties and tumorigenic potential of glioblastoma stem cells (GSCs) may translate into important therapeutics against these malignant tumors. GSCs preferentially express the nonreceptor tyrosine kinase bone marrow X-linked kinase (BMX), which maintains the stem cell-like phenotype and tumorigenic potential by activating STAT3 signaling and regulating the expression of critical GSC transcription factors. These data suggest that BMX represents a potential target amenable to specific disruption for the development of effective therapeutics against GSCs to improve treatment of GBM.

the microenvironment to promote tumor angiogenesis, immune evasion, and resistance to current therapies (Bao et al., 2006a, Bao et al., 2006b; Calabrese et al., 2007; Gilbertson and Rich, 2007; Liu et al., 2006; Wei et al., 2010). GSCs share some critical characteristics with normal neural stem/progenitor cells (NPCs), including expression of NPC markers and residing in certain niches in vivo (Calabrese et al., 2007; Gilbertson and Rich, 2007; Vescovi et al., 2006; Zhou et al., 2009). However, GSCs are also significantly distinct from NPCs in many aspects, including active proliferation, genetic abnormalities, tumor formation, aberrant gene expression, and differential hypoxia response in GSCs, suggesting that there are fundamental differences in the signaling pathways controlling stem cell properties between GSCs and NPCs. Identification of unique signaling regulators that control the phenotype and tumorigenic potential of GSCs might provide new avenues for developing effective therapeutics against GSCs to improve GBM treatment.

The signal transducer and activator of transcription (STAT) family of transcription factors provide important signaling nodes downstream of extracellular regulators including cytokines (Bromberg, 2002; Levy and Darnell, 2002). Among the STATs, STAT3 prominently contributes to cellular transformation and tumor maintenance (Bromberg et al., 1999; Chan et al., 2004; Grivnenkov et al., 2009), particularly in GBM (de la Iglesia et al., 2009; Lo et al., 2008). A functional contribution of STAT3 in glioma growth was demonstrated in a number of studies (Dasgupta et al., 2009; Rahaman et al., 2002). Activation of a STAT3-mediated transcription network was recently associated with mesenchymal GBM transformation and poor patient survival (Carro et al., 2010). In addition to these effects, silencing STAT3 in GBM cells induces cellular differentiation (Li et al., 2009b), suggesting a role for STAT3 in maintaining an undifferentiated cellular state in tumors. Indeed, recent reports showed that STAT3 is constitutively activated in GSCs and that targeting STAT3 disrupts GSC maintenance (Sherry et al., 2009; Wang et al., 2009).

The bone marrow X-linked kinase (BMX, also known as ETK) is an intracellular nonreceptor tyrosine kinase that associates with and activates STAT3 (Saharinen et al., 1997; Tsai et al., 2000). BMX is a member of the Tec-family kinases characterized by the presence of several critical domains including the Plekstrin homology (PH), the Tec homology (TH), the Src homology SH3 and SH2, and the SH1 kinase domain (Smith et al., 2001). This kinase is activated by several chemokines, tumor necrosis factor receptor 2, vascular endothelial growth factor receptors, ErbB3, and integrins (Chen et al., 2001; Jiang et al., 2007; Pan et al., 2002). BMX is expressed in prostate cancer and hepatocellular carcinomas and its overexpression induced prostate neoplasia and skin hyperplasia (Dai et al., 2006; Guo et al., 2007; Paavonen et al., 2004). Inactivation or inhibition of BMX kinase markedly attenuates cell proliferation and impairs Src-induced cell transformation (Paz et al., 2005). In addition, BMX contributes to cancer cell resistance to radiation and chemotherapy (Guo et al., 2010; Xue et al., 1999), a phenotype associated with GSCs (Bao et al., 2006a; Liu et al., 2006). However, BMX has not been investigated in GSCs. Based on the functional importance of STAT3 in GSCs, we investigated the potential role of BMX in STAT3

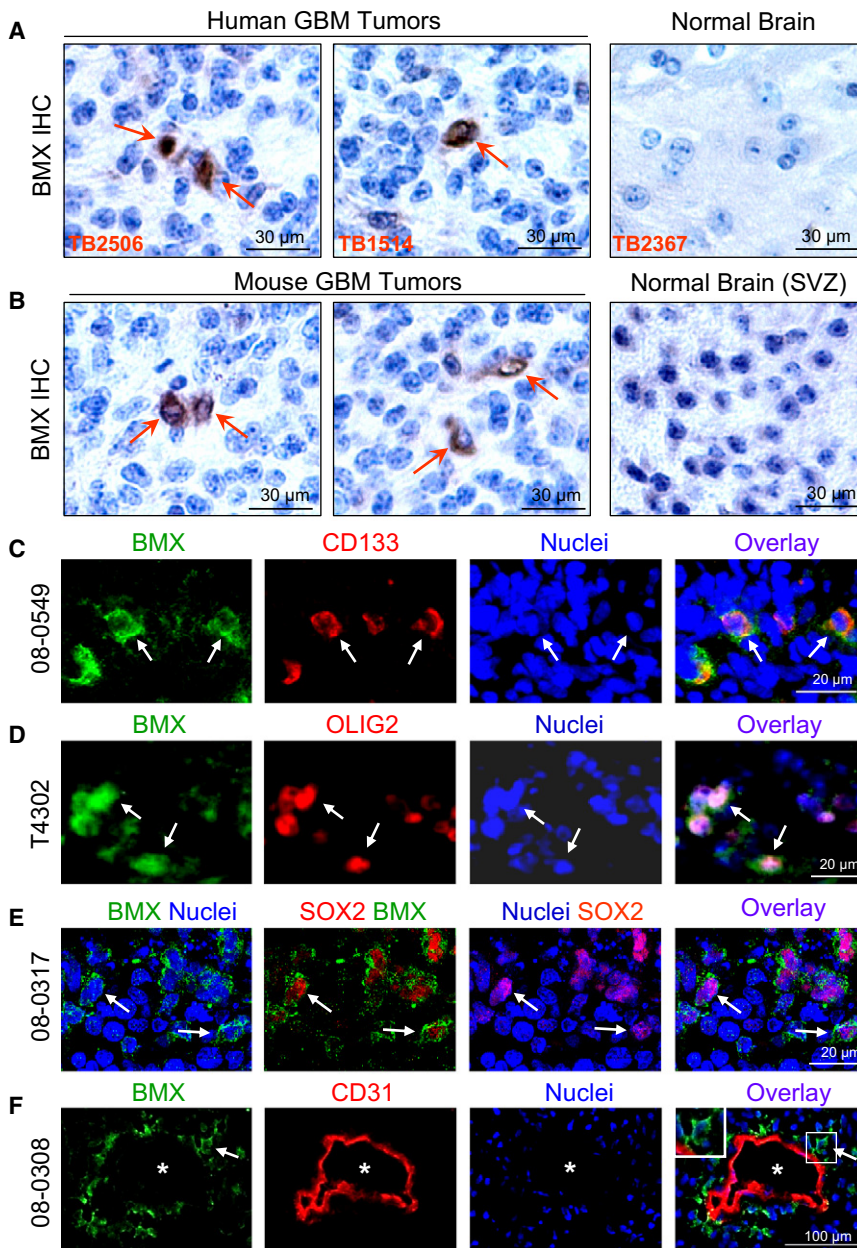
activation and the maintenance of stem-like phenotypes in GSCs.

## RESULTS

### BMX Is Differentially Expressed in GSCs Relative to Nonstem Tumor Cells and NPCs

To assess whether BMX is expressed in a fraction of cancer cells in primary GBM tissues, we performed immunohistochemical (IHC) staining on tissue arrays containing 36 human GBMs and 6 normal brain tissues. BMX was expressed in a subpopulation of tumor cells in 32 of 36 (88.8%) GBMs but none of normal brain tissues (Figure 1A; see Figure S1A and Table S1 available online). In addition, BMX was strongly expressed in a subpopulation of cancer cells in all seven examined mouse GBMs from a well characterized genetically engineered glioma model generated through the overexpression of PDGF in Nestin<sup>+</sup> cells (Hambardzumyan et al., 2009) (Figure 1B; Figure S1B). BMX expression was not detectable in matched normal mouse brain tissues including the subventricular zone (SVZ) that contains NPCs (Figure 1B; Figure S1B). As the frequency of BMX-positive [BMX(+)] cells was similar to that of GSCs in many studies, we performed immunofluorescent (IF) staining of BMX and GSC markers on frozen sections of GBM xenografts or surgical specimens (known genetic alterations in some GBMs are listed in Table S2). BMX was localized to glioma cells expressing the GSC markers CD133, OLIG2, and SOX2 (Figures 1C–1E), suggesting that BMX is preferentially expressed in GSCs in GBMs. In addition, some BMX(+) cells are proximal to blood vessels marked by anti-CD31 staining for endothelial cells in GBMs (Figure 1F; Figure S1C). Quantification showed that a greater fraction of BMX(+) cells reside in the close proximity (<5  $\mu$ m) to the blood vessels relative to BMX(–) cells and the average proximity of BMX(+) cells to endothelial cells is less than that of the BMX(–) cells (Figures S1D–S1F). These data support the preferential expression of BMX in GSC subpopulations that are often located in perivascular niches in GBMs (Calabrese et al., 2007).

To address whether BMX plays a role in GSCs, we further examined BMX expression in matched GSCs and nonstem tumor cells isolated from GBM surgical specimens or xenografts. BMX protein levels were elevated in GSCs enriched for the CD133 surface marker relative to matched nonstem tumor cells derived from five GBMs (Figure 2A, top panels). As BMX is a kinase, we validated the activation of BMX in GSCs by measuring its specific phosphorylation on Tyr<sup>40</sup> [pBMX(Y40)] (Figure 2A, middle panels). Because the utility of CD133 as a marker for enriching GSCs is not uniform (Beier et al., 2007), we examined BMX expression in GSCs enriched for CD15 (stage specific embryonic antigen-1, SSEA-1), which is an alternative GSC enrichment marker (Son et al., 2009). BMX expression was also elevated in CD15-enriched GSCs from two GBM specimens that did not express CD133 (Figure 2B). BMX was coexpressed with several GSC markers (OLIG2, SOX2, and CD133) (Figures 2C–2E; Figure S2). In addition, IHC staining confirmed more expression of BMX in GSCs than nonstem tumor cells isolated from GBM tumors (Figure 2F). Collectively, these data demonstrate that BMX is differentially expressed in GSCs relative to nonstem tumor cells.



**Figure 1. Expression of BMX in a Subpopulation of Cancer Cells Expressing Stem Cell Markers in GBMs**

(A) Immunohistochemical (IHC) staining of BMX in human primary GBMs and normal brain tissues. BMX(+) cells (indicated by arrows) are shown in brown. Sections were counterstained with hematoxylin to show nuclei.

(B) IHC staining of BMX in GBMs from a genetically engineered mouse model and normal mouse brains including the subventricular zone (SVZ). BMX(+) cells (brown) are indicated by arrows.

(C–E) Immunofluorescent (IF) staining of BMX and the GSC markers CD133 (C), OLIG2 (D), and SOX2 (E) on frozen sections of GBMs. BMX was labeled in green, CD133, OLIG2, or SOX2 in red. Nuclei were counterstained with DAPI (blue). Cells with extensive overlap are marked with arrows.

(F) IF staining of BMX(+) cells (green) in relation to blood vessels marked by CD31 staining for endothelial cells (red) in GBMs. Nuclei were counterstained with DAPI (blue). Asterisk denotes vessel lumen.

See also Figure S1, Table S1, and Table S2.

that SVZs express the NPC marker SOX2 but not BMX (Figure 3A). To insure that the expression patterns in mouse NPCs extended to human NPCs, we compared BMX expression in six GSC populations and five NPC lines. Consistent with our IHC studies, no NPC line expressed BMX whereas all six GSC populations expressed high levels of BMX protein (Figures 3B–3D; Figures S3E–S3G). GSCs and NPCs expressed their common markers SOX2, OLIG2, Nestin, and CD133 as expected (Figures 3B–3D; Figures S3F and S3G). As GBMs may also be lineage related to astrocytes, we further examined BMX expression in human and mouse astrocytes, and detected minimal BMX expression in these cells (Figures S3H–S3J). Taken together, these data demonstrate that BMX is preferentially expressed in GSCs

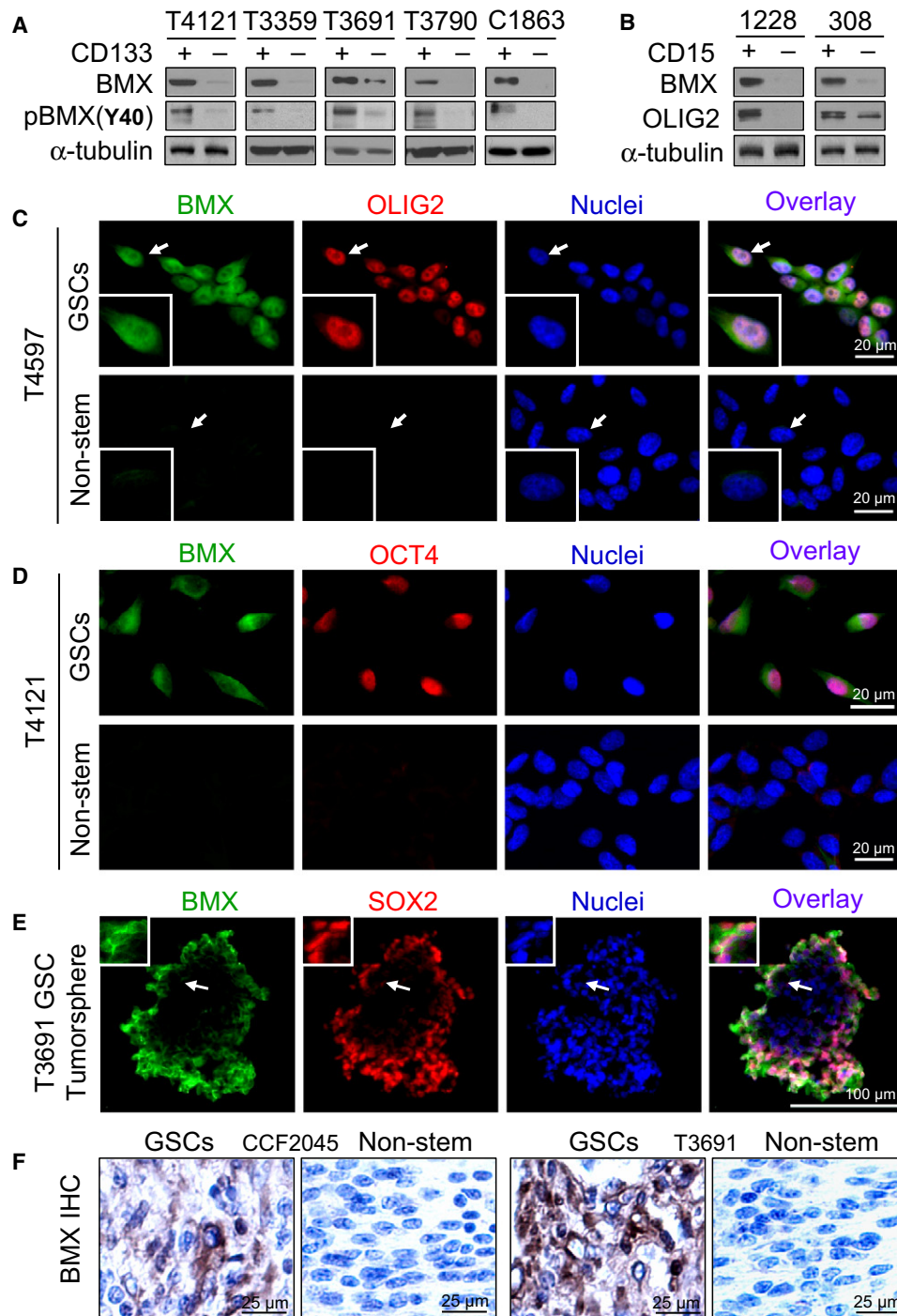
but not in NPCs or astrocytes. Thus, BMX may be a tractable GSC target due to its differential expression in neoplastic but not normal stem/progenitor cells.

### BMX Is an Upstream Activator of STAT3 in GSCs but Not in NPCs

As BMX regulates cellular physiology through activation of STAT3 (Saharinen et al., 1997; Tsai et al., 2000), the elevated BMX expression in GSCs may functionally link to STAT3 hyperactivation in GSCs. To define the role of BMX in regulating STAT3 activation in GSCs, we targeted BMX expression using two short hairpin RNAs (shRNAs) directed against nonoverlapping regions of BMX mRNA and then measured STAT3 activation in GSCs and NPCs. BMX knockdown in

As GSCs share many characteristics with NPCs, we investigated the relative expression levels of BMX in GSCs and NPCs. We first interrogated two mouse gene expression databases for the developing embryo (Edinburgh Mouse Atlas; <http://www.emouseatlas.org/emage/>) and the brain (Allen Institute Brain Atlas database; <http://mouse.brain-map.org>) and found that BMX was rarely expressed in normal tissues during mouse development (Figure S3A) or any region of mouse brain (Figure S3B). We then isolated NPCs from brains of adult and developing mice and confirmed that BMX was not expressed in NPCs and mouse brains at any stage (Figures S3C and S3D). As NPCs are located in the SVZ germinal zones, we directly examined BMX expression by immunofluorescent (IF) staining in the developing and adult mouse brains and confirmed





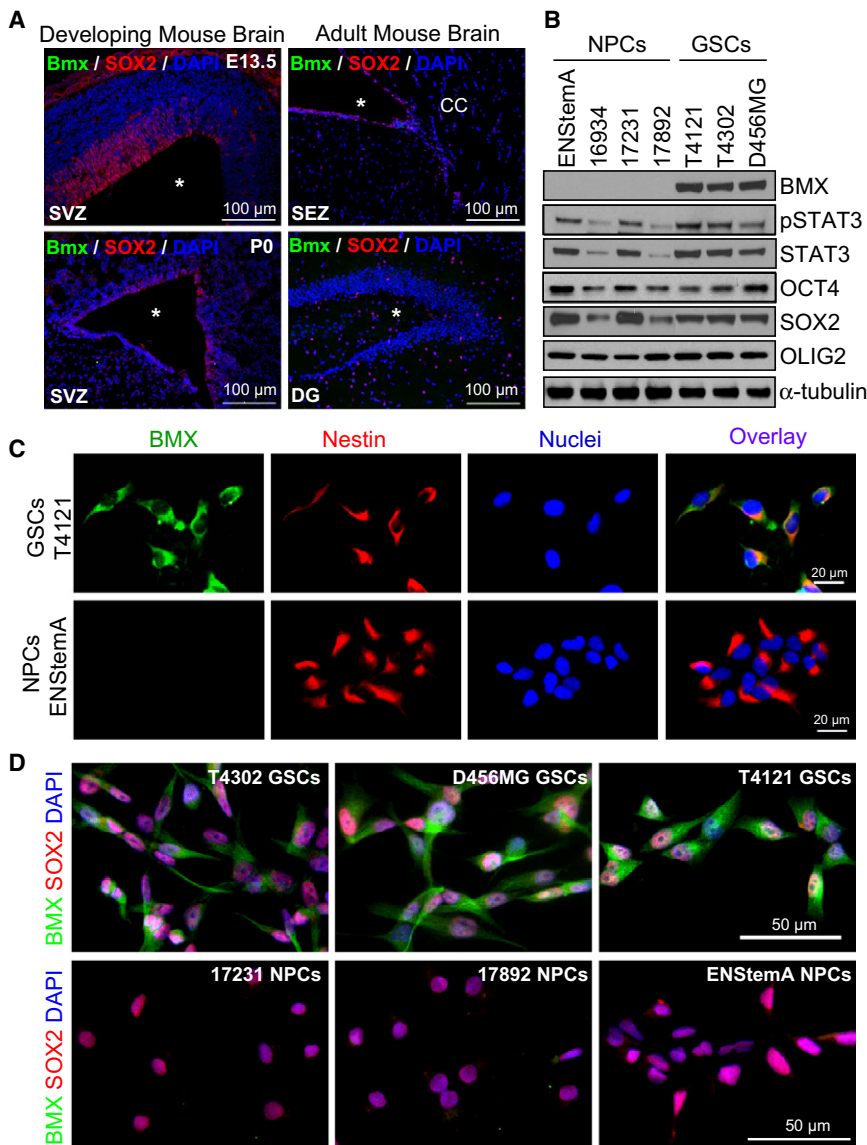
**Figure 2. Differential Expression of BMX in GSCs Relative to Nonstem Tumor Cells**

(A) Immunoblot (IB) analysis of BMX protein and its activating phosphorylation [pBMX(Y40)] in CD133-enriched GSCs and matched CD133-depleted tumor cells derived from five GBMs.

(B) IB analysis of BMX and the GSC marker OLIG2 in CD15-enriched GSCs and CD15-depleted tumor cells from two GBMs.

(C–E) IF staining of BMX with several GSC markers including OLIG2 (C), OCT4 (D), and SOX2 (E) in GSCs. BMX was labeled in green, OLIG2, OCT4, or SOX2 in red; and nuclei were counterstained with DAPI (blue). GSCs were cultured as attached monolayers (C) and (D) or tumorspheres (E).

(F) IHC staining of BMX in cultured GSCs and matched nonstem tumor cells (Nonstem) from a primary GBM (CCF2045) and a xenograft (T3691). See also Figure S2.



**Figure 3. Preferential Expression of BMX in Human GSCs but Not in NPCs and Subventricular Zone (SVZ) of Mouse Brains**

(A) IF staining of BMX in SVZs, subependymal zones (SEZs), and dentate gyrus (DG) in developing and adult mouse brains. Mouse brains were stained for BMX (green) and a NPC marker SOX2 (red). Nuclei were counterstained with DAPI (blue). \*, ventricles; CC, corpus callosum.

(B) IB analyses of BMX, STAT3, pSTAT3(Y705), OCT4, SOX2, and OLIG2 expression in three GSC populations and four human NPC lines.

(C) IF staining of BMX (green) and the NPC marker Nestin (red) in GSCs (T4121) and NPCs (ENStemA). Nuclei were counterstained with DAPI (blue).

(D) IF staining of BMX (green) and SOX2 (red) in three GSC populations and three human NPC lines (bottom panels). Nuclei were counterstained with DAPI (blue). See also Figure S3.

tional link between BMX expression and GSC maintenance was indicated by a marked decrease in BMX levels during GSC differentiation induced by serum or withdrawal of growth factors (Figures 5A and 5B; Figures S5A–S5D). To elucidate the role of BMX in GSC maintenance, we examined the effects of BMX knockdown on GSC proliferation and self-renewal. GSCs transduced with shBMX proliferated at lower rate than GSCs with the nontargeting (NT) control shRNA (Figure 5C; Figure S5E). In contrast, identical shBMXs had little to no effect on matched nonstem tumor cells (Figure 5D; Figure S5F) and human NPCs (Figure 5E; Figure S5G). Moreover, targeting BMX in GSCs reduced the efficiency of neurosphere formation (Figures 5F–5H and Figures S5H–S5J), a standard *in vitro*

GSCs reduced activating phosphorylation of STAT3 [pSTAT3 (Y705)] (Figures 4A and 4B; Figures S4A and S4B). In addition, BMX knockdown in CD15-enriched GSCs reduced activated STAT3 (Figure S4C). In contrast, BMX shRNA (shBMX) did not alter STAT3 activation in NPCs (Figures 4A and 4B; Figure S4A), despite high levels of basal activation of STAT3. We complemented these findings using a gain-of-function study. Ectopic expression of a constitutively active BMX (Flag-BMX-C) (Wu et al., 2001) increased STAT3 activation in GSCs but not NPCs (Figure 4C). These data demonstrate that BMX plays a pivotal role in STAT3 activation in GSCs but not in NPCs.

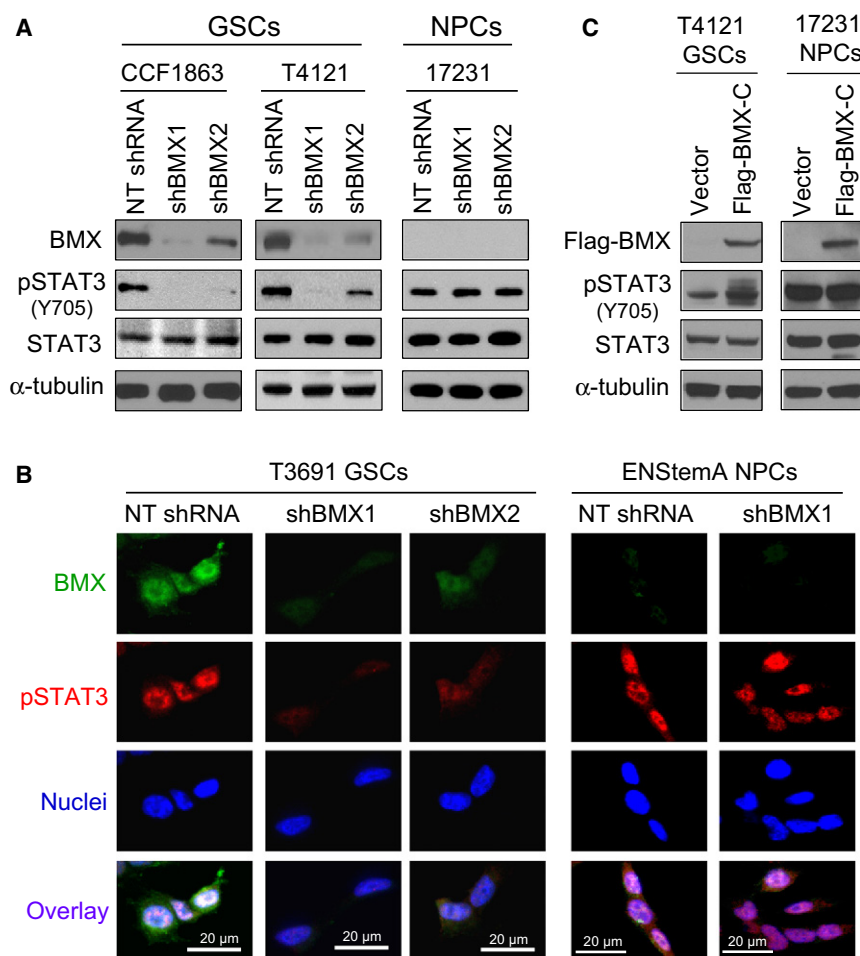
#### Disruption of BMX Impairs GSC Proliferation and Self-Renewal

As BMX is preferentially expressed in GSCs to promote STAT3 activation, and STAT3 mediates important signals for maintaining GSCs (Sherry et al., 2009; Wang et al., 2009), we interrogated the requirement of BMX in GSC maintenance. The initial func-

assay to assess the self-renewal and proliferation capacity of GSCs. In contrast, targeting BMX had no impact on neurosphere formation of NPCs (Figure 5I). To further confirm the effect of targeting BMX on GSCs, we introduced a dominant-negative BMX (BMX-DN) with a kinase-dead mutation (Tsai et al., 2000) into GSCs. Expression of BMX-DN in GSCs also inhibited GSC growth *in vitro* (Figure 5J) and suppressed neurosphere formation (Figures S5K and S5L). These data demonstrate that targeting BMX specifically disrupted GSC maintenance.

#### Targeting BMX Suppresses GSC Tumor Growth and Increases Survival of Mice Bearing Intracranial GBM Xenografts

The most important property of GSCs is their potent ability to propagate tumors *in vivo*. To address the requirement for BMX in maintaining the tumorigenic potential of GSCs, we examined the effects of BMX downregulation on tumor propagating capacity of GSCs. GSCs transduced with shBMX or NT shRNA



**Figure 4. BMX Is Required for STAT3 Activation in GSCs but Not in NPCs**

(A) IB analysis of activating phosphorylation of STAT3 (Y705) and total STAT3 in GSCs derived from the indicated GBMs and NPCs with BMX knockdown by shBMX1 or shBMX2. NT shRNA, nontargeting control shRNA.

(B) IF staining of phosphorylated STAT3 [pSTAT3 (Y705)] in GSCs and NPCs with BMX knockdown. BMX was labeled in green, pSTAT3 in red, and nuclei were counterstained with DAPI (blue).

(C) IB analysis of STAT3 activating phosphorylation and total STAT3 in GSCs and NPCs with ectopic expression of a constitutively active BMX with a Flag tag (Flag-BMX-C). See also Figure S4.

overexpression of BMX-DN in GSCs significantly increased the survival of mice bearing GSC-derived GBM tumors (Figure 6E). Taken together, these data demonstrate that BMX is required for maintaining the tumorigenic capacity of GSCs in vivo, suggesting BMX might be a useful therapeutic target.

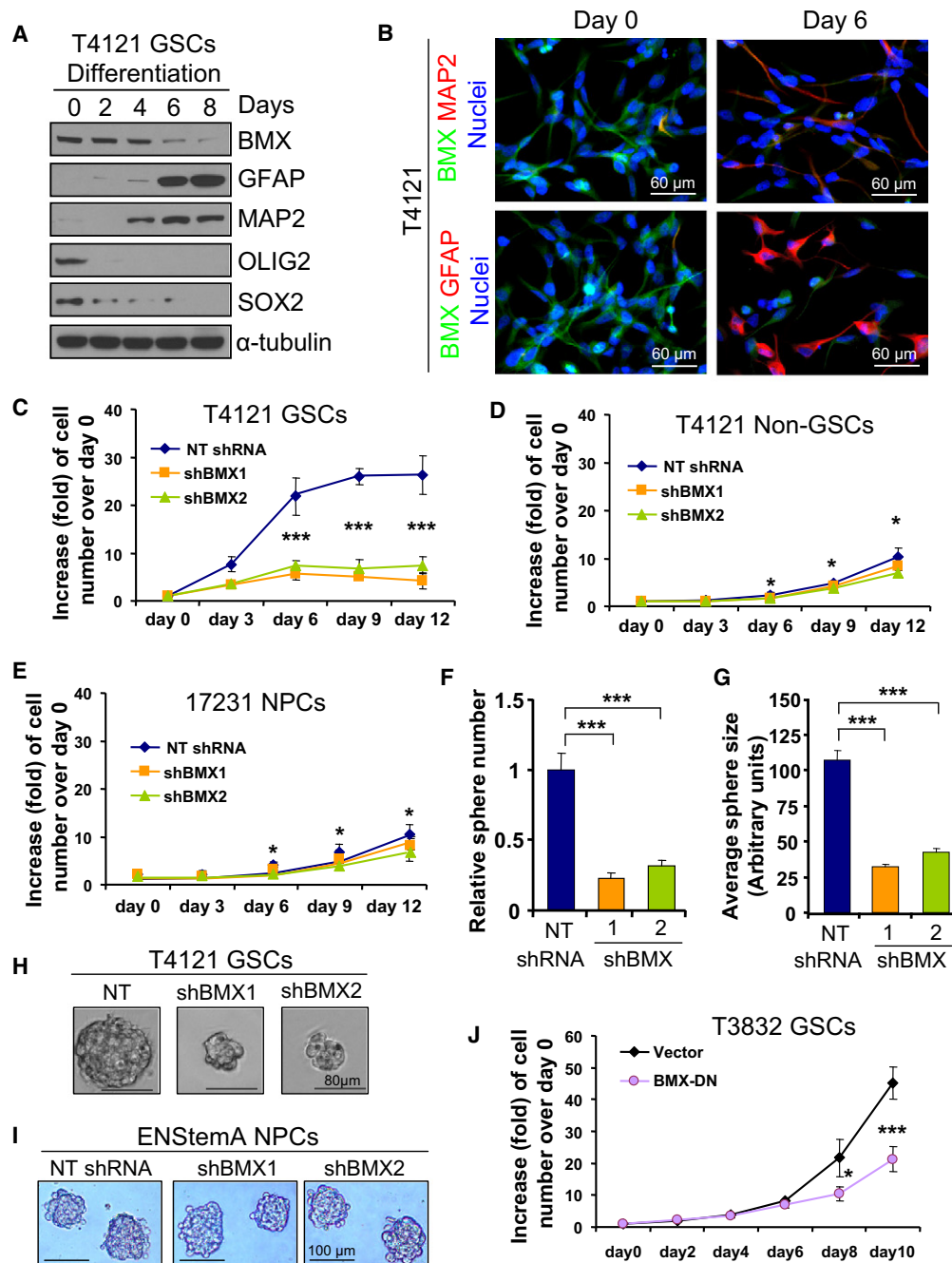
#### BMX Is Required to Maintain Expression of Stem Cell Transcription Factors in GSCs but Not in NPCs

As BMX is critical to maintain GSC phenotype in vitro and tumorigenic potential in vivo, BMX may control GSC properties regulated by key stem cell transcription factors (SCTFs). Therefore, we examined

were transplanted into the brains of immunocompromised mice. Animals bearing GSCs expressing shBMX displayed reduced tumor formation and increased tumor latency and survival relative to those bearing GSCs expressing NT shRNA (Figures 6A–6D). Necropsy of animals sacrificed simultaneously 32 days (T4121 GSCs) or 36 days (CCF2170 GSCs) after implantation revealed that GSCs transduced with shBMX frequently failed to generate tumors or have reduced tumor size relative to GSCs expressing NT shRNA (Figure 6A). Histological analyses confirmed that tumors developed and invaded into brains implanted with GSCs expressing NT shRNA (Figure 6B, left panels), whereas no tumor or only small tumor nodules developed in the brains implanted with GSCs transfected with shBMX (Figure 6B, right panels) at these early time points. The expression of BMX in a subpopulation of cancer cells in GBM tumors derived from GSCs expressing NT shRNA was confirmed by IHC staining (Figure 6C). Moreover, mice intracranially implanted with the GSCs transduced with shBMX survived significantly longer than those with GSCs transfected with NT shRNA (Figure 6D). Although >90% of cells were successfully targeted with shRNAs (data not shown), we found that BMX was still expressed in the tumors with delayed growth derived from the GSCs infected with shBMX lentiviruses (Figure S6), suggesting that the tumors that grew after BMX targeting had escaped knockdown. In addition,

the impact of BMX knockdown on the expression of a panel of critical SCTFs commonly expressed by GSCs. SOX2, OLIG2, OCT4, and NANOG were all reduced after transduction with shBMX in GSCs (Figure 7A). ShBMX1 and shBMX2 displayed different knockdown efficiencies that correlated with the extent of effects on GSC transcription factor expression (Figure 7A, left panels). These results were confirmed by IF staining of BMX and OLIG2 in GSCs. ShBMXs decreased OLIG2 protein levels in GSCs with greater efficiency in response to the more potent shRNA, shBMX1 (Figure 7B), suggesting that downregulation of these SCTFs by BMX knockdown is dose-dependent and specific. In contrast, introduction of shBMXs into NPCs had no effect on the expression of these key SCTFs (Figure 7A, right panels), indicating that expressional regulation of these transcription factors by BMX was specific to GSCs not NPCs. These results are consistent with the data above showing that BMX knockdown has little effect on STAT3 activation in NPCs (Figures 4A and 4B; Figure S4A). To further confirm the effect of targeting BMX on expression of SCTFs in GSCs, we introduced the BMX-DN into GSCs. Expression of BMX-DN in GSCs reduced STAT3 activation and expression of GSC transcription factors (Figure 7C). Furthermore, ectopic expression of constitutively active BMX in D456MG nonstem tumor cells, which had been shown to grow tumor in mouse brains when





**Figure 5. BMX Level Declines during GSC Differentiation and BMX Disruption Decreases GSC Proliferation and Tumorsphere Formation**

(A) IB analysis of BMX expression during GSC differentiation. Protein levels of BMX, SOX2, and OLIG2 (GSC transcription factors), GFAP (astrocyte marker), and MAP2 (neuronal marker) were examined during GSC differentiation induced by serum (10% FBS) at indicated times.

(B) IF staining of BMX (green) and the neuronal marker MAP2 (red) or the astrocyte marker GFAP (red) from day 0 to day 6 during GSC differentiation induced by serum. Nuclei were counterstained with DAPI (blue).

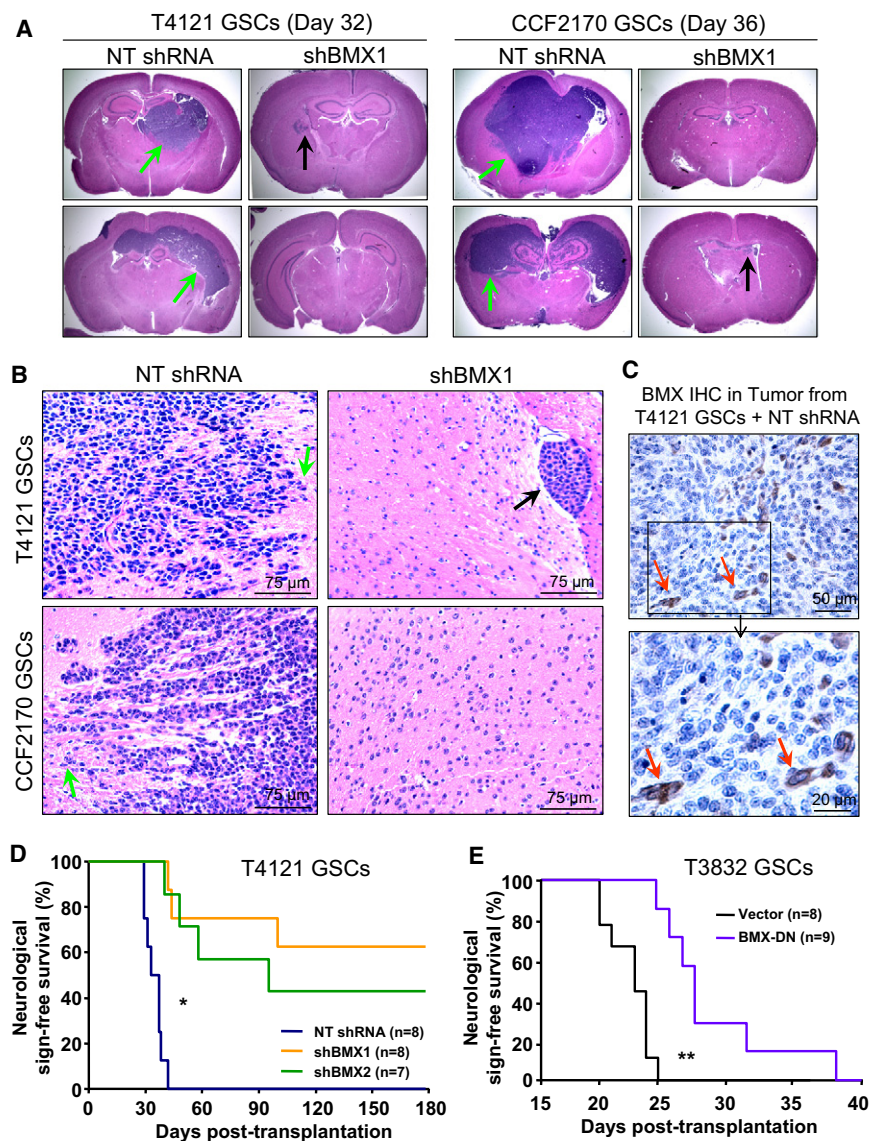
(C–E) Effects of BMX knockdown with shBMX1 or shBMX2 on cell proliferation in GSCs (C), matched nonstem tumor cells (D), and human NPCs (17231) (E). Cells ( $1 \times 10^4$  per well) transduced with shBMX or NT shRNA were plated in quadruplicate wells in stem cell media, and then counted at the indicated times (day 0–day 12). Data are means  $\pm$  standard deviation (SD) ( $n = 4$ ). \* $p < 0.05$ ; \*\*\* $p < 0.001$ .

(F–H) Effects of BMX knockdown by shBMX1 or shBMX2 on GSC tumorsphere formation. Representative images of tumorspheres derived from GSCs expressing NT shRNA, shBMX1, or shBMX2 are shown (H). Quantification shows reduced GSC tumorsphere number (F) and size (G) by shBMXs. Data are means  $\pm$  SD ( $n = 3$ ). \*\*\* $p < 0.001$ .

(I) Effect of shBMX1 or shBMX2 on neurosphere formation of NPCs. The representative images of neurospheres derived from NPCs expressing NT shRNA, shBMX1, or shBMX2 are shown.

(J) Effects of a dominant-negative BMX (BMX-DN) on cell proliferation of GSCs. Data are means  $\pm$  SD ( $n = 4$ ). \* $p < 0.002$ ; \*\*\* $p < 0.001$ .

See also Figure S5.



**Figure 6. Targeting BMX in GSCs Suppressed Tumor Growth and Increased Survival of Animals Bearing Intracranial GBMs**

Effects of targeting BMX on GBM tumor growth and animal survival were examined in the intracranial xenograft model. GSCs from indicated GBMs were transduced with NT shRNA, shBMX1, BMX-DN, or vector control through lentiviral infection, and then intracranially transplanted into the brains of immunocompromised mice ( $5 \times 10^3$  cells per mouse). Mouse brains implanted with GSCs expressing NT shRNA or shBMX were harvested simultaneously to examine the impact of BMX disruption on GBM tumor growth (A–C). In the animal survival experiments (D) and (E), mice implanted with GSCs expressing NT shRNA, shBMX, BMX-DN, or vector control were maintained until the development of neurological signs or for 180 days.

(A) Representative images of cross sections (hematoxylin and eosin [H&E] stained) of mouse brains harvested on day 32 (T4121 GSCs) or day 36 (CCF2170 GSCs) after transplantation of GSCs expressing NT shRNA or shBMX. Arrows indicate large tumors in the brains with GSCs expressing NT shRNA, or small tumors in the brains with GSCs expressing shBMX.

(B) Histological analysis of brain tumors derived from GSCs expressing NT shRNA or shBMX. Green arrows indicate cancer cells invading into normal tissue in brains implanted with GSCs expressing NT shRNA. A black arrow indicates a small tumor nodule in the brain implanted with GSCs expressing shBMX1.

(C) IHC staining of BMX in GBM xenografts derived from GSCs expressing NT shRNA. Arrows indicate the representative BMX(+) cells (brown). Sections were counterstained with hematoxylin.

(D) Kaplan-Meier survival curves of mice implanted with GSCs expressing shBMX1, shBMX2, or NT shRNA. \* $p < 0.001$ .

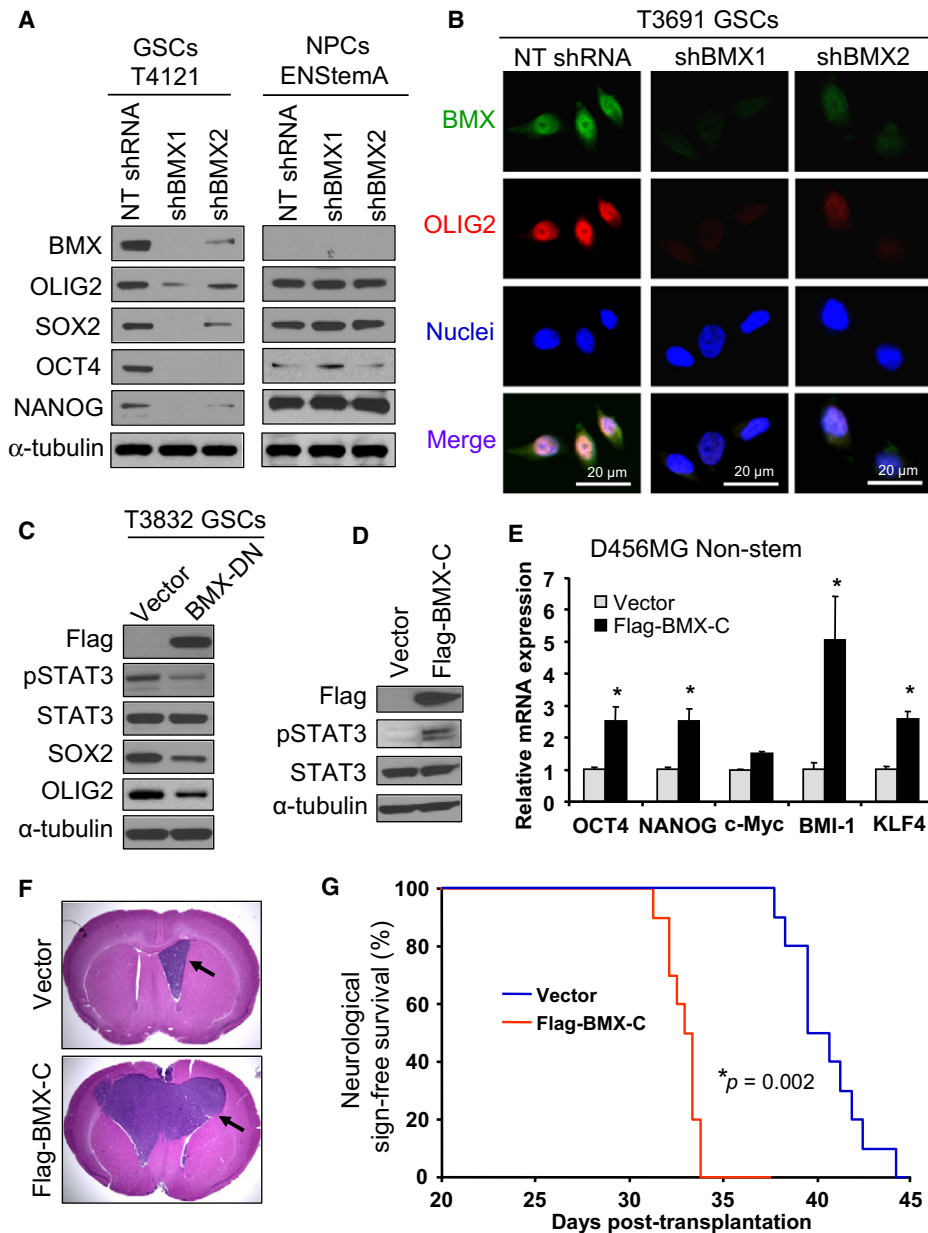
(E) Kaplan-Meier survival curves of mice implanted with GSCs transfected with a dominant-negative BMX (BMX-DN) or vector control. \*\* $p < 0.001$ . See also Figure S6.

a large number of cells were transplanted (Bao et al., 2006b), increased STAT3 activation (Figure 7D), induced expression of GSC transcription factors including NANOG, BMI1, KLF4, and OCT4 (Figure 7E), enhanced in vivo tumor growth (Figure 7F), and significantly reduced the survival of mice bearing these cells (Figure 7G), suggesting that BMX expression may promote the acquisition of cancer stem cell phenotypes. In contrast, ectopic expression of active BMX in NPCs did not enhance the high baseline activation of STAT3 and expression of SCTFs (Figure S7A) and neurosphere formation (Figure S7B). BMX overexpression alone in NPCs did not cause oncogenic transformation or tumor formation in vivo (data not shown). Taken together, our data demonstrate that BMX preferentially regulates the expression of key SCTFs in GSCs but not in NPCs, suggesting that the signaling pathway controlling the “stemness” might be different between GSCs and NPCs despite shared similar stem cell properties.

### Expression of a Constitutively Active STAT3 in GSCs Rescues the Effects Caused by BMX Downregulation

To confirm STAT3 activation as the critical molecular mechanism mediating BMX effects on expression of key SCTFs and the maintenance of GSC phenotypes, we examined whether ectopic expression of a constitutively active STAT3 (STAT3-C) (Bromberg et al., 1999) rescued the phenotypes caused by BMX downregulation. GSCs derived from primary GBMs were transduced with Flag-tagged STAT3-C (STAT3-C-Flag) or EGFP (enhanced green fluorescent protein) vector control. Expression of STAT3-C-Flag in GSCs not only restored the growth and proliferation but also rescued the impaired tumorsphere formation caused by BMX knockdown (Figures 8A–8D). Moreover, ectopic expression of STAT3-C-Flag restored the expression of key SCTFs reduced by BMX knockdown (Figure 8E), suggesting that BMX controls expression of these transcription factors through STAT3 activation. As BMX knockdown in GSCs significantly





**Figure 7. BMX Regulates Expression of Stem Cell Transcription Factors in GSCs but Not in NPCs**

(A) IB analysis of stem cell transcription factors (SCTFs) in GSCs and NPCs with BMX knockdown by shBMX1 or shBMX2. Cell lysates from the indicated GSCs and NPCs expressing shBMX1, shBMX2, or NT shRNA were analyzed for protein levels of BMX, OLIG2, OCT4, SOX2, and NANOG.

(B) IF staining of OLIG2 (a GSC transcription factor) in GSCs expressing NT shRNA, shBMX1, or shBMX2. BMX was labeled in green, OLIG2 in red. Nuclei were counterstained with DAPI (blue).

(C) IB analysis of STAT3 activating phosphorylation (pSTAT3-Y705) and expression of GSC transcription factors SOX2 and OLIG2 in GSCs expressing a dominant-negative BMX (BMX-DN).

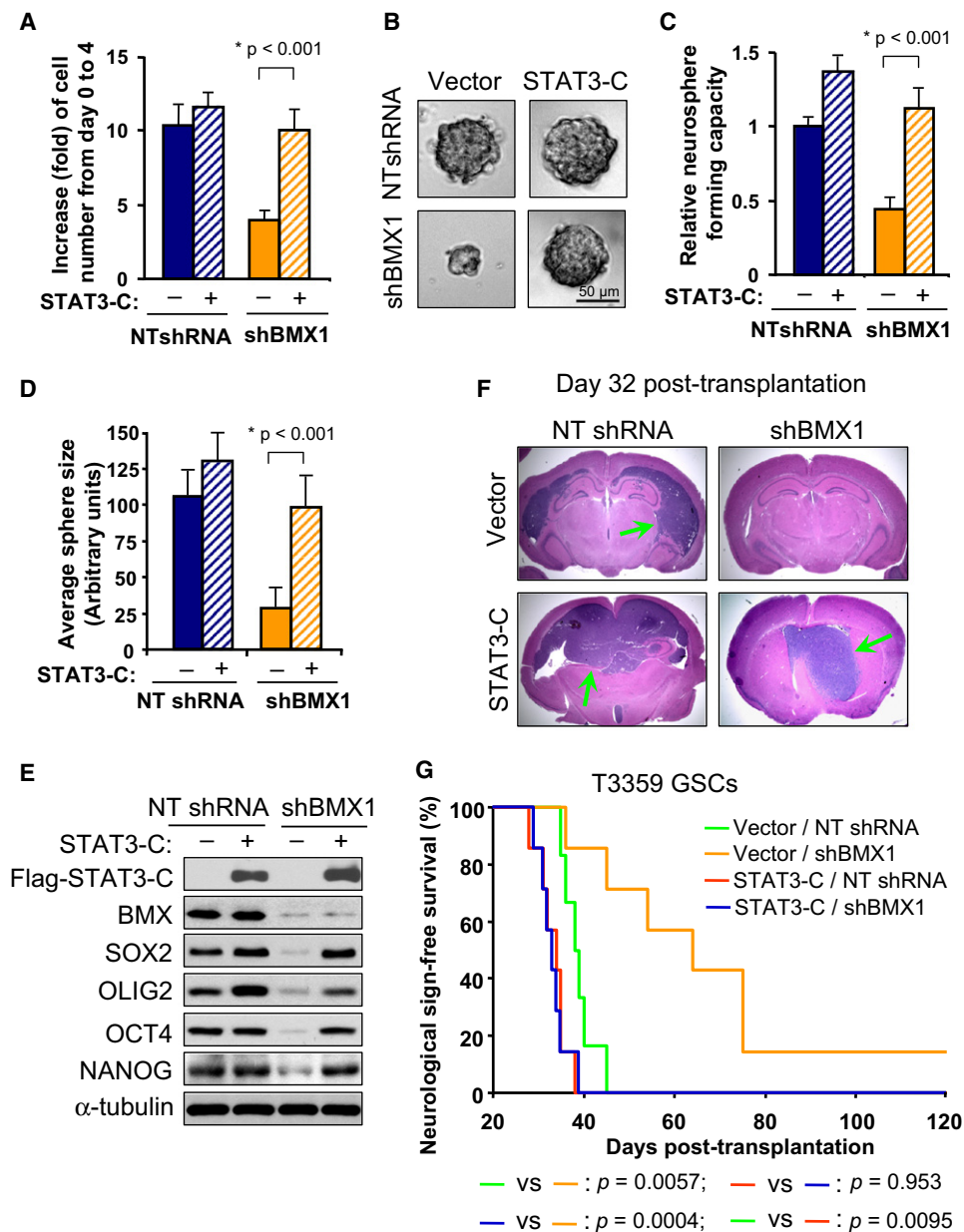
(D) IB analysis of STAT3 activation (pSTAT3-Y705) by expression of a constitutively active BMX (Flag-BMX-C) in D456MG nonstem tumor cells.

(E) RT-PCR analysis of mRNA expression of SCTFs including BMI-1, NANOG, OCT4, and KLF4 in D456MG nonstem tumor cells expressing the active Flag-BMX-C. Data are means  $\pm$  SD (n = 3). \*p < 0.002.

(F) Effect of the active Flag-BMX-C on tumor growth of GBM xenografts derived from D456MG nonstem tumor cells. D456 nonstem tumor cells were transduced with Flag-BMX-C or vector control, and then transplanted into the brains of immunodeficient mice via intracranial injection ( $2 \times 10^6$  cells per mouse). The brains were harvested simultaneously on day 42 after cell transplantation. The representative images of brain cross sections (H&E stained) are shown. Tumors inside brains are indicated by arrows.

(G) Kaplan-Meier survival curves of mice implanted with D456MG nonstem tumor cells transfected with the active Flag-BMX-C or control vector. D456 nonstem tumor cells transduced with Flag-BMX-C or control vector were intracranially transplanted into brains of immunodeficient mice ( $2 \times 10^6$  cells per mouse). The mice were maintained until the development of neurological signs or for 180 days. \*p = 0.002 by log rank survival analysis.

See also Figure S7.



**Figure 8. Ectopic Expression of a Constitutively Active STAT3 in GSCs Rescued the Phenotypes Caused by BMX Downregulation**

(A) Effects of a constitutively active STAT3 (STAT3-C-Flag) on cell proliferation of GSCs expressing NT shRNA or shBMX. GSCs (CCF2045) were transduced with STAT3-C-Flag or vector control and then targeted with shBMX1 or NT shRNA. The relative increase (folds) of cell number from day 0 to day 4 among the four groups (STAT3-C/Vector and shBMX1/NTshRNA) was compared. Data are means  $\pm$  SD (n = 5). \*p < 0.001.

(B–D) Effects of STAT3-C-Flag on tumorsphere formation of GSCs expressing NT shRNA or shBMX. Representative images of GSC tumorspheres derived from GSCs transduced with STAT3-C-Flag or vector and targeted with shBMX1 or NT shRNA are shown (B). Quantification shows the effects of STAT3-C-Flag expression on tumorsphere number (C) and size (D) of GSCs expressing NT shRNA or shBMX. Data are means  $\pm$  SD (n = 4). \*p < 0.001.

(E) IB analysis of GSC transcription factors (SOX2, OLIG2, OCT4, and NANOG) in CCF2045 GSCs transduced with STAT3-C-Flag or control vector in combination with shBMX or NT shRNA.

(F) Effects of STAT3-C expression on tumor growth of GSCs expressing shBMX or NT shRNA. GSCs (T3359) were transduced with STAT3-C-Flag or vector control, and then infected with shBMX1 or NT shRNA lentiviruses. 48 hr after infection, cells were transplanted into the brains of immunocompromised mice. Representative images of cross sections (H&E stained) of mouse brains from the indicated experimental groups harvested 32 days posttransplantation are shown. Arrows indicate tumors in mouse brains.

(G) Kaplan-Meier survival curves of mice implanted with GSCs expressing STAT3-C or vector in combination with shBMX or NT shRNA. Four groups of mice implanted with the GSCs were maintained until the development of neurological signs or for 180 days. The statistical analyses (p value) between each group are shown under the figure.

inhibited GBM tumor growth and increased animal survival, we examined whether expression of STAT3-C restored the GSC tumor growth. Forced expression of STAT3-C rescued in vivo GSC tumor growth impaired by BMX downregulation (Figure 8F) and attenuated the increased survival of mice bearing GSC-derived tumors expressing shBMX (Figure 8G). Expression of the STAT3-C in GSCs abolished the effects of shBMX on tumor growth in vivo. These data demonstrate that BMX signals through STAT3 to maintain the stem cell phenotype and tumorigenic potential of GSCs.

## DISCUSSION

Despite recent advances in cancer therapies, patients diagnosed with GBM have had little improvement in their survival over the past decades. The explanations for the failure of GBM therapies are numerous, but a newly appreciated source of complexity in tumor response is the presence of GSCs that permit sustained tumor propagation. Recent studies support the concept that many cancers, including GBMs, contain cellular hierarchies in which not all cancer cells have equal potential to propagate or maintain tumors (Gilbertson and Rich, 2007; Lobo et al., 2007; Rosen and Jordan, 2009). Thus, biologic screens of cancer stem cells (CSCs) may yield effective pharmacologic therapies (Gupta et al., 2009; Zhou et al., 2009; Frank et al., 2010). Optimal development of specific agents against CSCs may involve targeting critical regulators or signaling pathways not shared by normal somatic stem cells, to prevent unwanted toxicities to normal stem cells. In this study, we demonstrate that the nonreceptor tyrosine kinase BMX activates STAT3 signaling in GSCs but not in NPCs, indicating that BMX may represent one such unique target for the development of anti-GSC therapeutic agents. As BMX is preferentially expressed in GSC population that is only a small fraction of total cancer cells within a GBM tumor, an analysis of BMX expression based on gene expression profile of whole tumor tissues is unlikely to be informative. As current expression databases such as Oncomine, TCGA and REMBRANDT describe the average characteristics of expression profile of tumor tissues from glioma patients, interrogation of these databases did not provide a conclusive indication regarding the association of BMX expression and patient survival.

We have found that BMX regulates STAT3 activation in GSCs but not in NPCs and plays essential roles in maintaining the GSC phenotype and tumorigenic potential. Through STAT3 signaling, BMX controls the expression of key SCTFs, including SOX2, OLIG2, NANOG and OCT4 in GSCs. A recent study demonstrated that sufficient activation of Jak-STAT3 signaling is critical for reprogramming to ground state pluripotency and essential for the maintenance of embryonic stem cells (Yang et al., 2010). Our data suggest that the upstream activator of STAT3 signaling important for maintaining stem cell phenotypes may differ between normal and neoplastic stem cells. The preferential expression of BMX in GSCs and its role in maintaining GSC phenotypes through STAT3 activation suggests that BMX kinase may be a druggable target for GSCs. Although STAT3 is considered to be a potentially important signaling node in CSCs, it is difficult to inhibit specifically as STAT3 serves important functions in normal tissue homeostasis. In contrast, BMX has several

attractive aspects as a molecular target. First, kinases are relatively amenable to targeting with low molecular weight inhibitors. Clinical trials of kinase inhibitors (generally directed against receptor tyrosine kinases) for GBM treatment have been largely negative (reviewed in Sathornsumetee and Rich 2007), but these inhibitors failed in GBMs due to redundancy in receptor binding and signaling to intracellular mediators (Stommel et al., 2007). As an intracellular signal transducer that integrates upstream signaling, BMX may be attractive as its signaling may be more specific in GSCs. Several signaling pathways feed into the activation of STAT3 in GSCs, including Notch, IL-6 (interleukin-6) and PI3K (phosphoinositide-3-kinase) (Wang et al., 2009; Fan et al., 2010). A recent study from our group demonstrated that GSCs preferentially express both IL-6 coreceptors (IL6R $\alpha$  and gp130) and targeting IL6R $\alpha$  expression reduced STAT3 activation and inhibited GSC survival and tumor growth (Wang et al., 2009). Thus, it is enticing to believe that IL6R-mediated signaling may activate BMX that in turn activates STAT3. Although the upstream kinase that directly activates BMX in GSCs has not been defined, BMX mediates critical activation of STAT3 in GSCs suggesting that functional disruption of BMX may attenuate multiple key signals involved in CSC growth and survival. The toxicity of BMX inhibitors may be modest as BMX is not expressed in NPCs and normal brain, and it is dispensable in normal development and survival, as demonstrated by a minimal phenotype in a knockout mouse model (Rajantie et al., 2001).

CSCs are not cell autonomous but reside in defined functional niches (Calabrese et al., 2007; Gilbertson and Rich, 2007; Lathia et al., 2010). Previous studies suggested that BMX can additionally mediate other effects associated with the GSC phenotype: therapeutic resistance, invasion and angiogenesis. BMX expression has been linked to resistance to chemotherapy in prostate and lung carcinoma cells (Guo et al., 2010; Xue et al., 1999). BMX also facilitates integrin-mediated motility through interactions with focal adhesion kinase (Chen et al., 2001), but direct validation of a role of BMX in GSC-mediated cancer invasion is yet to be determined. In addition, BMX may play an important role in angiogenesis. Limb ischemia in BMX knockout mice led to reduced recovery and angiogenesis, whereas BMX transgenic mice overexpressing BMX displayed an accelerated angiogenic response (He et al., 2006). BMX activation also stimulates VEGF production (Chau et al., 2005). Thus, systemic inhibition of BMX may not only directly target GSC self-renewal and growth but may also disrupt the functional vascular niches that are essential for GSC maintenance (Calabrese et al., 2007; Gilbertson and Rich, 2007; Li Z et al., 2009). As we have demonstrated that GSCs are an important source of VEGF (Bao et al., 2006b), it is likely that BMX contributes to the ability of GSCs to support angiogenesis as well. Inhibiting BMX in conjunction with anti-angiogenic drugs (particularly VEGF antagonists) or conventional radiation and/or chemotherapeutic therapies may achieve elimination of GSCs with benefits against the tumor bulk. Thus, BMX may offer antitumor activity with acceptable toxicity. It is unlikely that anti-CSC therapies will be effective in isolation, so the potential effects of BMX inhibition on angiogenesis and therapeutic resistance may offer synergies. In conclusion, our studies laid an essential foundation for the development of BMX inhibitors as an anti-GSC therapy.



## EXPERIMENTAL PROCEDURES

## Isolation and Culture of Glioma-Derived Cells

GSCs and nonstem tumor cells were isolated and characterized from GBM xenografts or surgical specimens as previously described (Bao et al., 2006a; Li et al., 2009a). De-identified GBM specimens were collected for this study from Cleveland Clinic Brain Tumor and Neuro-Oncology Center in accordance with an Institutional Review Board-approved protocol, and informed consent was obtained from all GBM patients contributing tumor specimens. The cancer stem cell phenotype of GSCs was confirmed by functional assays of self-renewal (serial neurosphere passage), stem cell marker expression, differentiation induction, and tumor propagation. Genetic changes of glioma cells were determined by fluorescent in situ hybridization (FISH).

## Immunoblot Analysis and Immunofluorescent Staining

Immunoblot (IB) analysis and immunofluorescent (IF) staining of cells and tissues sections was performed as described (Bao et al., 2006a; Lathia et al., 2010). Specific antibodies against BMX (ab59360, ab59288, Abcam; or BD), phospho-BMX (Y40) (Cell Signaling), phospho-BMX-pY566 (ab59409, Abcam), CD133/1 (Miltenyi or Abcam), STAT3 and phospho-STAT3 (pSTAT3-Y705) (Cell Signaling), SOX2 (RnD or Millipore), OLIG2 (RnD), Nestin (Abcam), GFAP and MAP2 (Sigma-Aldrich or Covance), NANOG (Millipore), TRA-1-85 (RnD), Flag (M2, Sigma-Aldrich), OCT4 and  $\alpha$ -tubulin (Millipore or Santa Cruz) were used for IB analysis or IF staining.

## Immunohistochemistry

Immunohistochemistry (IHC) staining of tumor and normal tissue sections was done with an ABC kit using DAB (3,3'-Diaminobenzidine) detection (Vector Lab) as previously described (Bao et al., 2006b). Two purified anti-BMX antibodies (#610792, BD; ab59288, Abcam) were identified to stain specifically on paraffin embedded sections. Tissue arrays containing 36 human GBM surgical specimens and 6 normal brain tissues were provided by Dr. McLendon from Duke Brain Tumor Center. The genetically engineered mouse GBM tumors (the RCAS-PDGF Nestin-TVA system) were provided by Dr. Hambardzumyan in our department.

## Differentiation Assay

GSCs were cultured on Matrigel-coated coverslips or dishes and induced for differentiation through withdrawal of EGF and bFGF growth factors or by addition of serum (10% FBS in  $\alpha$ -MEM). At indicated time points, cells were harvested for immunoblot analysis or fixed for IF or IHC staining as described above.

## Human Neural Progenitor Cells and Astrocytes and the Isolation of Neural Progenitor Cells and Astrocytes from Mouse Brains

Four human neural progenitor cell (NPC) lines (15167, 16934, 17231, and 17896 derived from fetal brains, Lonza) and one human embryonic stem cell-derived NPC line (ENStemA, Millipore) were cultured and maintained in suspension culture according to vendor's instruction or propagated on the BD stem cell Matrigel-coated dishes or coverslips in supplemented Neurobasal stem cell media. Human astrocytes (NHA) were also obtained from Lonza. Murine NPCs were isolated from mouse brains and characterized as described (Rietze and Reynolds, 2006). Mouse NPCs were isolated from wild-type C57BL/6 mouse embryos and neonatal mice by dissociation of the harvested forebrains with papain and DNaseI. Adult NPCs were derived from 7- to 10-week-old C57BL/6 mice by microdissection of the periventricular region then digestion by papain and trypsin. Isolated NPCs were plated in suspension culture in stem cell media and allowed to grow through three passages to ensure self-renewal before harvesting for immunoblot analysis or plating on coverslips for IF staining.

## DNA Constructs and Lentiviral Transfection

Lentiviral clones expressing shBMX or NT shRNA (SHC002) were acquired from Sigma-Aldrich. Two of five shBMXs (shBMX1 and shBMX2) that displayed high knockdown efficiency (80%–90% reduction) were used for all related experiments. A lentiviral construct expressing constitutively active BMX (Flag-BMX-C) was generated by cloning a DNA fragment corresponding to BMX residues 243–675 into the XbaI and Sall sites of pLCMV-Flag-neo

vector (a gift of Dr. P. Chumakov at Cleveland Clinic) in frame with the N-terminal Flag sequence. A point mutation leading to Lys-to-Gln substitution in the active site of the kinase domain (corresponding to position 445 of BMX protein) was introduced into the Flag-BMX-C construct to generate a dominant negative BMX (BMX-DN). Mutagenesis was done using QuickChange Multi III Site-Directed Mutagenesis kit (Stratagene) and confirmed by sequencing. Viral particles were produced in 293T cells with the pPACK set of helper plasmids (System Biosciences) in stem cell media. Viral stocks were concentrated by precipitation with PEG-8000 and titered according to the manufacturer's instructions.

## Rescue Experiments with the Constitutively Active STAT3

A constitutively active STAT3 (STAT3-C; Bromberg et al., 1999) retroviral construct was generated by cloning the STAT3-C with a C-terminal Flag tag into HindIII site within the pLEGFP-N1 vector (BD Biosciences). For rescue experiments, GSCs were transduced with STAT3-C-Flag retroviral construct or pLEGFP control vector, and allowed to recover for 48 hr. Neomycin-resistant cells were selected by exposure to G418 for 7 days. Stable cells expressing STAT3-C-Flag or EGFP control were transduced to express either shBMX or NT shRNA via lentiviral infection. 48 hr postinfection, cells were plated to assess cell proliferation, self-renewal, and expression of stem cell factors, or used for in vivo experiments.

## Proliferation and Neurosphere Formation Assays

Cell proliferation and neurosphere/tumorsphere formation were measured as previously described (Li Z et al., 2009; Lathia et al., 2010). All data were normalized to day 0 and presented as mean  $\pm$  standard deviation.

## Quantitative RT-PCR

Total cellular RNA was isolated with an RNeasy kit (QIAGEN) and reverse transcribed into cDNA using the Superscript III Kit (Invitrogen). Real-time PCR was performed on an Applied Biosystems 7900HT cyclor using SYBR-Green Mastermix (SA Biosciences) with the following primers: OCT4: forward 5'-GAGAACCAGAGTGAGAGGCAACC-3' and reverse 5'-CATAGTCGCTGCTTGATCGCTTG-3'; NANOG: forward 5'-AATACCTCAGCCTCCAGCAGATG-3' and reverse 5'-TGCCTCACACCATTTGCTATTCTTC-3'; c-MYC: forward 5'-TC AAGAGGCGAACACACAAC-3' and reverse 5'-GGCCTTTTCATTGTTTCC A-3'; BMI1: forward 5'-CCAGGGCTTTTCAAAAATGA-3' and reverse 5'-GCA TCACAGTCATTGCTGCT-3'; KLF4: forward 5'-CCCAATTACCCATCCTT CCT-3' and reverse 5'-AGGTTTCTCACCTGTGTGGG-3';  $\beta$ -actin: forward 5'-AGAAAATCTGGCACCACACC-3', and reverse 5'-AGAGGCGTACAGGGA TAGCA-3'.

## Intracranial Tumor Formation In Vivo

Intracranial transplantation of GSCs to establish GBM xenografts was performed as described (Bao et al., 2006a; Li Z et al., 2009). GSCs were transduced with NT shRNA or shBMX, and/or with BMX-DN or control vector through lentiviral infection twice at a 24-hr interval. Twenty-four hours after the second transduction, viable cells ( $5 \times 10^3$ /animal or as indicated) were engrafted intracranially into athymic/nude or NOG (NOD/Shi-scid/IL-2R $\gamma^{null}$ ) immunocompromised mice. For the survival experiments, animals were maintained until manifestation of neurological signs or for 180 days. To compare the tumor growth, mouse brains implanted with GSCs expressing shBMX or NT shRNA, BMX-DN, or control vector were harvested on same day as indicated after GSC implantation. All animal procedures conformed to the Cleveland Clinic IACUC-approved protocol.

## Statistical Analysis

All grouped data are presented as mean  $\pm$  standard deviation (SD). Difference between groups was assessed by one-way analysis of variance (ANOVA) or one-way ANOVA on ranks tests. All in vitro experiments were repeated at least three times. For the in vivo experiments, log rank survival analysis was performed. SigmaStat Software (Version 3.5) was used for all statistical analyses.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at doi:10.1016/j.ccr.2011.03.004.

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