Serine/Threonine Kinase MLK4 Determines Mesenchymal Identity in Glioma Stem Cells in an NF-κB-dependent Manner

Highlights
- Silencing MLK4 attenuates mesenchymal identity in glioma stem cells
- MLK4 activates NF-κB signaling by direct phosphorylation of IKKα
- MLK4 promotes radioresistance in GBM
- MLK4 expression negatively correlates with patient survival of MES but not PN GBM

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In Brief
Kim et al. show that stem-like cells of mesenchymal (MES) glioblastoma (GSCs) overexpress MLK4 and that silencing MLK4 reduces GSCs self-renewal, radioresistance, and tumorigenicity. IKKα is a MLK4 substrate and targeting the MLK4-driven NF-κB signaling could be a therapeutic strategy for MES glioblastoma.

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Serine/Threonine Kinase MLK4 Determines Mesenchymal Identity in Glioma Stem Cells in an NF-κB-dependent Manner

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SUMMARY

Activation of nuclear factor κB (NF-κB) induces mesenchymal (MES) transdifferentiation and radioresistance in glioma stem cells (GSCs), but molecular mechanisms for NF-κB activation in GSCs are currently unknown. Here, we report that mixed lineage kinase 4 (MLK4) is overexpressed in MES but not proneural (PN) GSCs. Silencing MLK4 suppresses self-renewal, motility, tumorigenesis, and radioresistance of MES GSCs via a loss of the MES signature. MLK4 binds and phosphorylates the NF-κB regulator IKKa, leading to activation of NF-κB signaling in GSCs. MLK4 expression is inversely correlated with patient prognosis in MES, but not PN high-grade gliomas. Collectively, our results uncover MLK4 as an upstream regulator of NF-κB signaling and a potential molecular target for the MES subtype of glioblastomas.

Significance

Proneural (PN) and mesenchymal (MES) glioma stem cells (GSCs) represent two mutually exclusive and biologically distinct GSC subtypes. GBM patients with the MES GSC signature belong to the poorer prognosis subclass and are resistant to irradiation. Identification of regulatory mechanisms that regulate MES transdifferentiation is therefore critical for developing GSC-targeted therapy. Here, we find that silencing MLK4 inhibits de novo and acquired (radiation-induced) MES GSCs both in vitro and in vivo. In addition, we present evidence of IKKa as a direct molecular target of MLK4 that drives NF-κB pathway activation, thereby promoting MES transdifferentiation of GSCs. Targeting the MLK4-driven NF-κB signaling axis could be a therapeutic strategy for GBM patients with a MES signature.
INTRODUCTION

Death in patients afflicted with cancer is mainly due to uncontrollable recurrence of the primary tumor following failure of current therapies. Glioblastoma (GBM) is one such cancer type wherein aggressive treatment strategies including surgery, ionizing radiation (IR), and chemotherapy provide only palliation (Chaichana et al., 2010; Goodwin et al., 2011; Kaper et al., 2006; Omuro and DeAngelis, 2013). Recent investigations have uncovered substantial phenotypic changes that occur in post-therapeutic cancers (Candolfi et al., 2012; Locatelli et al., 2013; Oh et al., 2012; Tafani et al., 2011).

Each GBM tumor is composed of heterogeneous tumor cell populations including those with stem cell properties, termed glioma-initiating cells or glioma stem cells (GSCs) (Hemmati et al., 2003; Singh et al., 2003, 2004). Accumulating evidence suggests that stem cell properties in cancers contribute to therapeutic resistance and cancer initiation (Bao et al., 2006; Beier et al., 2011; Capper et al., 2009; Vescovi et al., 2006). Thus, development of therapeutics targeting GSCs may provide considerable benefit to cancer patients.

In various cancers, transition from the epithelial to mesenchymal (MES) subtype is associated with advanced malignancy (epithelial-MES transition; EMT) (Nurwidya et al., 2012; Shirkoohi, 2013). We and others have identified an EMT-like phenotypic shift that occurs in GBM in response to factors in the microenvironment or cytotoxic treatments that is now termed proneural (PN) to MES transition (PMT) (Bhat et al., 2013; Halliday et al., 2014; Mao et al., 2013; Phillips et al., 2006). Of note, the MES identity is a hallmark of glioma aggressiveness and is strongly associated with the poor outcome of patients (Bhat et al., 2013; Carro et al., 2010). Despite the central role of PMT in brain tumor recurrence, little is known about the molecular mechanisms that control this phenotypic shift in GSCs. Advances in genome-wide, genetic, and genomic profiling have uncovered the aberrant signaling pathways that are essential for various phenotypes of cancers such as tumorigenesis, cellular proliferation, motility, and therapy resistance. Such oncogenic pathways include nuclear factor-κB (NF-κB) and its downstream targets (Fan et al., 2005, 2010; Ferris and Grandis, 2007; Yamamoto et al., 2013). In particular, we have recently demonstrated that NF-κB is a master regulator that causes MES transdifferentiation with an associated radioresistance in PN GSCs (Bhat et al., 2013).

Mixed lineage kinase 4 (MLK4) is a relatively poorly characterized serine/threonine kinase. While the genetic mutation of MLK4 is frequent in microsatellite unstable subtype of colorectal cancers (CRCs) that exhibit extremely poor prognosis compared with other subtypes, this genetic event is substantially rare in GBM (Martini et al., 2013). In CRCs, the role of MLK4 in intracellular signaling is associated with KRAS- and MAPK-mediated pathways (Marusiak et al., 2014). To date, it remains undetermined whether MLK4 plays a pathophysiological role in GBM and/or cancer stem cells. In this study, driven by the fact that MLK4 is a lead candidate from kinase screens, we seek to examine its role in gliogenesis, MES transdifferentiation, and radioresistance in GSCs.

RESULTS

MLK4 Is Overexpressed in MES GSCs

To identify protein kinases that are associated with the MES signature, we compared the genome-wide expression levels of 349 kinase-encoding genes in the patient-derived PN and MES GSC-containing primary cultures grown as neurospheres (hereafter designated as glioma spheres) using 18 PN and 12 MES high-grade glioma (HGG) patient-derived tumor specimens. We found six genes (MLK4, LYN, MST4, VRK2, PRKCH, and MAPK9) that were significantly upregulated in MES glioma spheres by 4-fold or more compared with PN spheres and/or somatic cell types including normal neural progenitors and astrocytes (Figure 1A). Upon silencing with the short hairpin RNA (shRNA) technique, MLK4 was the only gene that was required for survival of MES, but not PN, glioma spheres as judged by the increased number of cells in the sub-G1 phase of the cell cycle (Figures 1B and 1C). MLK4 was also the most highly expressed kinase in the MES glioma spheres when compared with normal neural progenitors or astrocytes (Figures 1D and 1E). By contrast, the other members of the MLK family did not show any noticeable difference in expression between PN and MES glioma spheres (Figure S1B). We validated the elevated MLK4 levels in MES glioma spheres by real-time PCR (Figure S1C), western blotting (Figure 1E), and immunofluorescence in HGG patient-derived glioma spheres (Figures 1F, S1D, Table S1, and S2). Moreover, co-expression of MLK4 with a MES marker, CD44, was confirmed using immunofluorescence and fluorescence-activated cell sorting (FACS) analysis in tumor spheres (Figures 1G, 1H, S1E, and S1F). Interestingly, MLK4 promoter was hypermethylated in PN tumors with isocitrate dehydrogenase 1 (IDH1) mutation, suggesting that epigenetic silencing could be one mechanism by which MLK4 is expressed at lower levels in PN GBMs (Figure S1G). Finally, we observed that MLK4 expression is enriched in the stemness-associated ALDEFUOR-positive subpopulations in MES glioma spheres (Figures 1I and 1J). We compared MLK4 expression between self-renewing undifferentiated MES glioma spheres and their sister cultures that underwent differentiation. At 2 days after differentiation induction, MLK4 mRNA levels rapidly declined with a sharp contrast with the marked induction of an osteocyte differentiation marker RUNX2 (Figure 1K). Taken together, these data indicate that MLK4 is overexpressed in the MES subtype of GSCs.

Silencing MLK4 Induces Apoptosis, and Reduces Sphere Formation and Tumorigenesis in MES Tumor Spheres

Next, we investigated the physiological role of MLK4 in MES GSCs using two shRNA lentiviral vectors targeting independent regions of the MLK4 gene in two well-characterized glioma sphere samples (83 and 267) (Bhat et al., 2013; Jeon et al., 2014; Mao et al., 2013). Western blotting showed more than 80% reduction of MLK4 by one of the MLK4 shRNA (shMLK4_2) in both MES 83 and 267 spheres (Figures S2A and S4C). In parallel, we generated MLK4 knockout (KO) cells by utilizing the CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeats-associated protein 9) (Figure S2B). Both shMLK4 silencing and MLK4 KO significantly attenuated...
the ability of in vitro clonal growth of MES 83 and 267 cells (Figures 2A and S2C). Reduction of sphere growth could be only partially attributed to diminished proliferation, since silencing MLK4 caused weak, yet significant, reduction of the proportion of 5-ethynyl-2’-deoxyuridine (EdU)-incorporated cells (Figure 2B). On the contrary, shMLK4-infected MES spheres, but not PN 528 glioma spheres, showed a substantial accumulation of apoptotic cells as judged by Annexin V/PI staining (Figures 2C and S2D). In addition, there was no significant difference in the effects of shRNA targeting for the other three MLK genes on sphere formation between PN and MES glioma cells (Figure S2E), suggesting MLK4 is unique in that it is preferentially required for the self-renewal and proliferation of MES glioma spheres in vitro, while MLK1-3 may have a general role for self-renewal and proliferation of multiple cell types. Consequently, a limiting dilution sphere-forming assay demonstrated that the proportion of self-renewing sphere-forming unit was largely reduced by both shMLK4 and MLK4 KO in these MES glioma cells (Figures 2D and S2F).

To test the in vivo consequence of silencing MLK4 in MES 83 and 267 glioma spheres, we used intracranial mouse models. MLK4 silencing significantly decreased in vivo tumor growth and/or extended median survival of mice without neurological deficit due to tumor burden compared with the control shNT-infected MES 83 glioma spheres (p = 0.0021 for shMLK4_1, p = 0.0003 for shMLK4_2; log-rank p value) and MES 267 glioma spheres (p = 0.0551 for shMLK4_1, p = 0.0013 for shMLK4_2;
log-rank p value). Of note, the more effective shRNA clone (shMLK4_2 versus shMLK4_1) had more impact on prolonged survival of mice (Figures 3A and 3B). These data indicate that MLK4 is required for both in vitro and in vivo growth of MES tumor spheres.

**MLK4 Is Required for the MES Phenotype in GSCs**

Aberrant activation of the MES phenotype in GBM is linked to increased cellular motility, glycolysis, and genes associated with wound healing and inflammatory properties (Zhong et al., 2010). Therefore, we investigated whether MLK4 inhibition influences these traits in MES glioma spheres from the single-cell resolution measurement. We performed time-lapse monitoring of individual cells from dissociated shMLK4 lentivirus-infected MES 83 spheres on micropatterned polydimethylsiloxane surfaces that mimic the fiber-like and/or conduit-like structures of in vivo parenchyma or stroma (Gallego-Perez et al., 2012). Traveling paths (tracks) of individual clones in each group displayed the clear difference of cellular motility in MES glioma sphere with shNT and shMLK4 infection (Figure 4A). Silencing MLK4 resulted in a significantly attenuated clonal motility in comparison with the control, determined by video time-lapse microscopy (Figure 4A, Movies S1, and S2).

We next investigated the glycolytic activity of shMLK4-infected MES glioma spheres. The extracellular acidification rate (ECAR) was measured as a glycolysis indicator. Diminished basal ECAR was observed in MES 83 spheres when they were infected with either one of the two shMLK4 lentiviruses but not shNT virus, and when mitochondrial respiration was blocked with oligomycin, compensatory glycolysis was even further diminished in these shMLK4-infected MES 83 spheres (Figure 4B).

Figure 2. Depletion of MLK4 Attenuates a Set of MES GSC Phenotypes

(A) Effects of MLK4 knockdown by shRNA (shMLK_1 or shMLK_2) and MLK4 knockout (KO) by CRISPR/Cas9 system on cell growth in MES 83 glioma spheres analyzed by AlamarBlue staining. Results were expressed as relative fluorescence units (RFU). Data are means ± SD (n = 6). ***p < 0.001.

(B) Representative images of EdU incorporation assays (left) and quantification of EdU-positive cells (right) in MES 83 glioma spheres expressing shNT or shMLK4. Cells in green represent EdU-positive cells. Nuclei were counterstained with DAPI (blue). Scale bar represents 20 μm. Data are means ± SD. *p < 0.05.

(C) FACS plots of Annexin V and PI staining in MES 83 and PN 528 glioma spheres expressing shNT, shMLK4_1, or shMLK_2.

(D) Effects of or shMLK4_2 and MLK4 KO on sphere-forming frequency of MES 83 glioma cells determined by limiting dilution assays. Stem cell frequency was calculated by extreme limiting dilution analysis. Data are means ± SD (n = 18). ***p < 0.001.

See also Figure S2.
We then asked whether MLK4 is required and/or sufficient for the expression of MES markers in GSCs. qRT-PCR showed that MLK4 knockdown markedly decreased the expression of MES genes (MET, WT1, BCL2A1, VIM, and SNAI1), whereas kinase active MLK4 (R470C) overexpression in PN 528 spheres resulted in the opposite effect (Figure 4C; Martini et al., 2013). A similar reduction of MES genes was also observed in MLK4 KO cells compared with controls (Figure S3B). As expected, gene-set enrichment analysis (GSEA) demonstrated signature global reduction of MES signature (Figure 4D). Interestingly, depletion of MLK4 in these MES glioma spheres did not induce a shift toward a PN gene signature (Figure S3C). Consistent with these mRNA expression data, the MES cell surface antigen CD44 was decreased by MLK4 knockdown or KO in MES 83 and 267 spheres, and in turn, MLK4 overexpression increased CD44 protein expression in PN 528 spheres (Figures 4E and S3D). The reduction of the MES markers was observed in xenograft tumors generated from shMLK4 lentivirus-infected MES 83 and 267 spheres as evidenced by VIM immunoreactivity, accompanied by retained MLK4 elimination (Figures 4F and S3E). Collectively, these results indicate that MLK4 could be a global regulator of the MES signature and traits associated with this subtype of tumors.

MLK4 Regulates NF-κB Signaling Axis in MES GSCs via Binding and Phosphorylation of IKKα

To gain insight into the mechanisms by which MLK4 control the MES signature, we first examined downstream pathways that MLK4 could potentially regulate. It has been reported that four MLK genes (MLK1–4) belong to the mitogen-activated protein kinase (MAPK) family that activate c-Jun amino-terminal kinase (JNK) and p38 as downstream targets (Gallo and Johnson, 2002). However, we observed no noticeable difference in ERK, JNK, and phospho-p38 levels in glioma spheres of either PN or MES subtype (Figures S4A and S4B). Given the molecular link between NF-κB pathway activation and MES differentiation

Figure 3. Silencing MLK4 in MES GSCs Suppressed Tumor Growth and Increased Mouse Survival

(A) Representative H&E staining of mouse brains harvested on day 15 (MES 83) or day 58 (MES 267) after transplantation of MES glioma spheres expressing shNT, shMLK_1, or shMLK_2. Scale bars represent 2 mm.

(B) Kaplan-Meier survival curves of mice intracranially transplanted with MES 83 or 267 glioma spheres that were infected with indicated shRNAs (n = 7). Tables show median survival of mice.
Figure 4. Mesenchymal Phenotypes in Glioma Spheres Are Dependent on MLK4

(A) Effects of MLK4 knockdown (shMLK4_1 or shMLK4_2) on the motility of MES 83 glioma spheres. Representative track pattern images of individual MES 83 cells are shown (upper). Quantification shows the effect of shMLK_1 or shMLK4_2 on single-clone migration velocity (lower). Scale bar represents 200 μm. Data are means ± SD. ***p < 0.001.

(B) Effects of MLK4 knockdown (shMLK4_1 or shMLK4_2) on the glycolysis of MES 83 spheres were measured by recording the extracellular acidification rate (ECAR) in a Seahorse Bioanalyzer. Data are means ± SD. **p < 0.01.

(C) qRT-PCR analysis for gene expression of MES markers including MET, WT-1, BCL2A1, VIM, and SNAI1 in MES 83 glioma spheres expressing shNT or shMLK4 and in PN 528 glioma spheres expressing vector or kinase active mutant of MLK4 (R470C). Data are means ± SD (n = 3). **p < 0.01; ***p < 0.001.

(D) GSEA plot of TCGA MES gene signatures in shNT or shMLK4 of glioma spheres. Gene expression profile data are obtained by cDNA microarray using with MES glioma spheres expressing shNT or shMLK4. Gene expression profile data are obtained by cDNA microarray using with MES glioma spheres expressing shNT or shMLK4. Gene sets for MES GBM defined by the TCGA MES signature were used for this analysis [Verhaak et al., 2010]. The normalized enrichment scores (NES) is shown in the plot.
we examined whether this pathway is altered in response to modulation of MLK4. Western blotting showed MLK4 silencing reduces phosphorylated form, but not the total form, of the upstream regulators of the NF-κB pathway, IKKα and IKKβ (Figure 5A). Luciferase assay using an NF-κB-responsive element demonstrated that MLK4 knockdown considerably reduces the NF-κB transcriptional activity in MES 83 glioma spheres. Whole cell lysates as input positive control and pull-down with immunoglobulin G as negative control.

In vitro kinase assay showing MLK4 phosphorylates IKKα, but not IKKβ, in a dose-dependent manner. After incubation with purified baculovirus-expressed IKKα or IKKβ and MLK4 recombinant proteins including ATP, immunoblot blot was performed with anti-p-serine antibody.

Effects of IKKα siRNA knockdown in PN 528 glioma spheres on MLK4-mediated NF-Gluc activity. Data are means ± SD (n = 3). **p < 0.01; ***p < 0.001.

GSEA showing NF-κB gene signature is reduced in MLK4-depleted MES glioma spheres. The normalized enrichment score (NES) is shown in the plot.

Limiting dilution neurosphere-forming assay in MES 83 glioma spheres with control vector or MLK4 (K151A) overexpression. Stem cell frequency was calculated by extreme limiting dilution analysis. ***p < 0.001.

Kaplan-Meier survival curves (K) and representative H&E staining images (L) of mice intracranially injected MES 83 glioma spheres with vector or MLK4 (K151A). Arrows indicate tumors. Scales bar represent 2 mm (upper) and 100 μm (lower).

Representative immunohistochemistry (IHC) imaging for CD44 and VIM in control vector- or MLK4 (K151A)-overexpressing MES 83 derived tumors. Scales bar represent 20 μm. See also Figure S4.
observed in the shMLK4 lysates compared with controls (Figure 5C). Immunoprecipitation and in vitro kinase analysis revealed that MLK4 physically interacts with both IKKα and IKKβ, but preferentially phosphorylates IKKα over IKKβ (Figures 5D, 5E, and S4D). To test the function of IKKα on MLK4-mediated NF-κB reporter activity, we used siRNA targeting IKKα and kinase dead IKKα (S176A) to repress their expression levels. MLK4-mediated NF-κB luciferase activity was strongly suppressed by knockdown of IKKα wild-type, but not IKKα mutant (S176A) (Figures 5F and S4E). Analysis of the microarray data with 30 glioma sphere samples showed that MLK4 expression is strongly correlated with the genes involved in the NF-κB pathway (Figures S4F and S4G). Furthermore, GSEA showed that genes affected by MLK4 silencing in MES glioma spheres were enriched for the NF-κB pathway signature (Figure 5G). These data implicate MLK4 as an upstream regulator of NF-κB signaling.

To evaluate whether the function of MLK4 in MES GSCs is dependent on its kinase activity, we carried out site-directed mutagenesis to create MLK4(K151A) mutant clone (Martini et al., 2013). Overexpression of MLK4(K151A) in MES 83 spheres inhibited cell proliferation and self-renewal activity, accompanied by a reduction of p-IKKα/β expression and NF-κB reporter activity (Figures 5H–5J and S4H). Furthermore, we found that IKKα exhibits strong association with MLK4-WT, whereas the association with MLK4(K151A) was relatively weak, indicating that MLK4-IKKα interaction is dependent, at least partially, on MLK4 phosphorylation (Figure S4I). We then investigated whether overexpression of this catalytically inactive MLK4 affects in vivo tumorigenic potential using the orthotopic xenograft models (Figures 5L and 5K). MLK4(K151A) overexpression in MES 83 spheres significantly decreased in vivo tumor growth, thereby prolonging the median survival of the tumor-bearing mice. Strikingly, central necrosis of tumors—one criterion for the diagnosis of Grade IV GBM—was not observed in xenografted tumors derived from MES glioma spheres overexpressing the K151A mutant form of MLK4, accompanied by reduced immunoreactivity to CD44 and VIM (Figure 5M). Together, these data suggest that MLK4 activates the NF-κB pathway via interaction and phosphorylation of IKKα in MES glioma spheres, and that the catalytic activity of MLK4 is essential for maintenance of HGG and the MES phenotype.

Following IR Therapy, PN GSC-Derived Brain Tumors Develop Sensitivity to MLK4 Elimination

We previously reported that IR to glioma spheres increases MES marker expression with a concomitant decrease in PN markers, raising a possibility that IR induces PMT in GSCs in vitro (Mao et al., 2013). To determine whether MLK4 plays a role in this process, we first examined the change of MLK4 expression in IR-treated PN glioma spheres (PN 157 and 84) by qRT-PCR. MLK4 was dramatically upregulated 24 hr after IR treatment, with an accompanied increase in MES marker CD44 at later time points, in these two PN glioma sphere samples (Figure 6A). In turn, when we combined MLK4 knockdown and IR treatment in MES 83 spheres, MLK4 silencing by shRNA almost completely abolished their IR-induced NF-κB activity (Figure 6B). Given that radioresistance of GBM tumors is linked to the MES subtype of GSCs (Bhat et al., 2013) and our data indicate the elevation of MLK4 by IR, we investigated the combined effect of MLK4 knockdown and IR treatment in mouse xenograft tumors using PN glioma spheres (GSC 23). Whole brain radiation was performed with four cycles of 2.5 Gy on consecutive days for these tumor-burdened mice to mimic the clinical regimen of radiation therapy for GBM. As expected, and consistent with our in vitro findings (Figure 2), silencing MLK4 alone did not noticeably affect the growth of these PN tumors, whereas IR prolonged the survival of these mice (Figures 6C and 6D). In these tumors, expression levels of nuclear p-p65, CD44, and VIM were strongly induced by IR, which, in turn, were markedly suppressed by shMLK4 (Figures S5A and S5B). As expected, combination of MLK4 inhibition with IR treatment showed a further reduction in their growth kinetics, and significantly prolonged mouse survival (median survival in GSC 23: shNT versus shNT + IR, 44 versus 60.5 days; shMLK4 versus shMLK4+IR, 44 versus 70 days; Figures 6C and 6D). In the MES glioma sphere-derived mouse xenograft models, the combined IR treatment and shMLK4 also prolonged mouse survival (median survival of shMLK4₁ versus shMLK4₁ + IR in MES 83: 27 versus 37 days; Figure S5C).

To investigate the role of MLK4 in promoting GBM radioresistance, we injected either the control vector- or MLK4-overexpressing PN 8–11 spheres intracranially into immunocompromised mouse brains. IR treatment extended the survival of the control vector-overexpressing PN sphere-derived tumor-bearing mice by 51.5 days (Figure 6E). In contrast, in MLK4-overexpressing tumor-bearing mice, IR extended the mouse survival for only 21 days (Figure 6E). These results indicate that expression of MLK4 is not only required, but sufficient to promote radioresistance in GBM.

MLK4 Expression Is Associated with Poor Survival of MES but Not PN GBM

Lastly, we investigated the clinical relevance of our findings using tissues obtained from GBM patients. We performed immunohistochemical staining of MLK4, OLIG2 (PN GBM marker), and CD44 (MES GBM marker) in 87 HGG specimens. As shown in Figure 7A, MLK4 expression strongly correlated with CD44 expression, whereas the staining of MLK4 and OLIG2 were mutually exclusive. Intriguingly, in the OLIG2 high HGG patients, the MLK4 expression level was not informative for post-surgical patient survival. In contrast, high-MLK4 patients displayed significantly shorter survival in the CD44 high patient group, indicating that MLK4 expression predicts survival of patients within the MES subgroup (Figure 7B). Following this exploratory study, we validated these findings in a larger cohort of cases (n = 108). Consistent with the results observed with the initial datasets, both the progression-free survival and overall survival of patients with CD44 high GBMs were significantly correlated with MLK4 expression, whereas those patients with OLIG2 high GBMs did not show any noticeable correlation (Figure 7C). Taken together, MLK4 is specifically upregulated in MES HGGs with CD44 expression and is negatively linked with post-surgical survival of patients with MES tumors.

DISCUSSION

In this study, we demonstrate that (1) MLK4 is highly expressed in glioma spheres derived from MES but not PN GBM; (2)
overexpression of MLK4 promotes, while MLK4 knockdown attenuates, a set of the MES glioma stem cell phenotypes; (3) MLK4 shRNA inhibits tumor initiation and propagation of MES GSCs in the mouse xenograft models; (4) radiation-induced MES transition in PN glioma spheres is associated with substantial upregulation of MLK4; (5) unlike the results of MLK4 knockdown alone, its combined treatment with IR in PN glioma sphere-derived tumors significantly attenuates tumor growth, thereby prolonging survival of tumor-burdened mice; (6) MLK4 binds and phosphorylates the NF-κB regulator IKKα, thereby activating the DNA binding of NF-κB in MES glioma spheres; and (7) MLK4 expression is highly elevated in CD44 high MES subtype HGGs, and is informative for the prognosis of patients with MES, but not PN, HGG/GBM.

One major finding in this study is the differential role of MLK4 in PN and MES GSCs. Glioma spheres derived from OLIG2 high tumors that were enriched with PN GSCs (Bhat et al., 2013; Mao et al., 2013) expressed substantially lower levels of MLK4 than MLK4 inhibition. Overexpression of MLK4 promoted, whereas its knockdown reduced, the expression of the MES markers, suggesting that MLK4 is not only required but most likely sufficient to induce the MES phenotype. In orthotopic models, although mice bearing shMLK4 GSCs showed improved survival, they eventually died due to tumor burden despite retaining MLK4 downregulation (Figure S3E). It is not clear whether the MES glioma spheres initially contain de novo MLK4-resistant cell population or whether they acquire resistance during tumor formation in vivo. Molecular and phenotypic characterization of these tumors is crucial to determine whether persistent MES identity independent of MLK4-mediated signaling mechanisms promotes tumor growth, or whether a shift of the MES phenotype to another subtype by shMLK4 subsequently leads to tumor growth despite MLK4 targeting. The frequency of MLK4 mutation in GBM is not very clear as yet. One recent report by Martini et al. (2013) showed that two out of nine cases had a missense/nonsense mutation. Our study revealed the transcriptional and

overexpression of MLK4 promotes, while MLK4 knockdown attenuates, a set of the MES glioma stem cell phenotypes; (3) MLK4 shRNA inhibits tumor initiation and propagation of MES GSCs in the mouse xenograft models; (4) radiation-induced MES transition in PN glioma spheres is associated with substantial upregulation of MLK4; (5) unlike the results of MLK4 knockdown alone, its combined treatment with IR in PN glioma sphere-derived tumors significantly attenuates tumor growth, thereby prolonging survival of tumor-burdened mice; (6) MLK4 binds and phosphorylates the NF-κB regulator IKKα, thereby activating the DNA binding of NF-κB in MES glioma spheres; and (7) MLK4 expression is highly elevated in CD44 high MES subtype HGGs, and is informative for the prognosis of patients with MES, but not PN, HGG/GBM.

One major finding in this study is the differential role of MLK4 in PN and MES GSCs. Glioma spheres derived from OLIG2 high tumors that were enriched with PN GSCs (Bhat et al., 2013; Mao et al., 2013) expressed substantially lower levels of MLK4 than MLK4 inhibition. Overexpression of MLK4 promoted, whereas its knockdown reduced, the expression of the MES markers, suggesting that MLK4 is not only required but most likely sufficient to induce the MES phenotype. In orthotopic models, although mice bearing shMLK4 GSCs showed improved survival, they eventually died due to tumor burden despite retaining MLK4 downregulation (Figure S3E). It is not clear whether the MES glioma spheres initially contain de novo MLK4-resistant cell population or whether they acquire resistance during tumor formation in vivo. Molecular and phenotypic characterization of these tumors is crucial to determine whether persistent MES identity independent of MLK4-mediated signaling mechanisms promotes tumor growth, or whether a shift of the MES phenotype to another subtype by shMLK4 subsequently leads to tumor growth despite MLK4 targeting. The frequency of MLK4 mutation in GBM is not very clear as yet. One recent report by Martini et al. (2013) showed that two out of nine cases had a missense/nonsense mutation. Our study revealed the transcriptional and
protein function of MLK4 in GBM and GSCs. Future studies will elucidate the genomic contribution of MLK4 on GBM.

The second finding in this study is the identification of a regulatory role for MLK4 in NF-κB signaling in GBM cells. We found that MLK4 interacts and phosphorylates IKKα in MES glioma spheres, thereby regulating its kinase activity. Through this interaction with IKKα, MLK4 controls the DNA binding activity of NF-κB (Figure 5D). Coupled with our previous study that NF-κB drives the MES signature by inducing master regulatory transcription factors (Bhat et al., 2013), the MLK4-NF-κB axis could be a key node in fine-tuning the MES phenotype in GSCs. The current study also uncovers MLK4 as a potential link between tumor radioresistance and PMT in GSCs. IR-induced NF-κB activation was dependent on MLK4, and therefore silencing MLK4 presumably attenuates PMT following IR treatment. To date there are no effective treatment options for recurrent GBMs after failure of the first-line therapies, and the identification of MLK4 presents an attractive target, inhibition of which could potentially improve patient outcome. Given the broad significance of the NF-κB pathway in a variety of cancers, future studies are required to explore the pathophysiological role of MLK4 in other cancers that depend on NF-κB for their growth and therapy resistance.

Unlike some other cancers (e.g. colon), virtually all GBMs are detected without prior pre-cancerous stage in the clinic, except for the minor population of the IDH mutant secondary GBMs. Therefore, whether GBMs initiate as PN tumors and evolve to gain more MES identity is an important question, but still remains unknown. Recent studies including our own indicate that PMT is likely associated with post-treatment reinitiation of tumors as recurrence (Chen et al., 2012). Indeed, some of newly diagnosed GBM tumors that arise as PN tumors reappear as MES tumors at recurrence (Phillips et al., 2006). Nonetheless, it is still debatable whether or not post-therapeutic PMT of GBM tumors is a universal phenomenon. In breast cancers, therapeutic insult appears to develop MES phenotypes, termed de novo-recurrence evolution (Mani et al., 2008; Moody et al., 2005). However, intratumoral sampling variations may mislead the data interpretation and are merely indicative of tumor heterogeneity. This open question aside, our data indicated that stimulation of PN glioma spheres with tumor necrosis factor α (TNF-α) upregulated MLK4 expression (data not shown). Previously we reported that TNF-α is derived from macrophages/microglia and is associated with the transcriptomic plasticity of the PN and MES states. It is known that the MES GBMs exhibit a high degree of
macrophages/microglial infiltration. Possibly, macrophages and microglia may provide extrinsic signals including TNF-α to cause MLK4 activation, thereby promoting PMT of tumors and/or GSCs via NF-κB activation (Bhat et al., 2013). Alternatively, or on top of these extrinsic signals, MLK4 activation in MES GSCs could be driven by intrinsic signals in GSCs. In recent reports, a set of core transcriptome signatures was identified to determine tumor evolutionary dynamics and subsequent cellular identities in cancers and GSCs (Hnisz et al., 2013; Suva et al., 2014). It is important to further elucidate how intrinsic signaling mechanisms and extrinsic microenvironment factors cooperate and compete to sustain the MES identity in GSCs and GBM tumors.

In the current neuro-oncology clinic, irradiation is a mainstay of post-surgical GBM treatment, and recent studies including ours elucidated that NF-κB is activated in post-IR tumors (Bhat et al., 2013; Brach et al., 1991). NF-κB is an essential molecule in various cancers for the regulation of cell proliferation, motility, therapy resistance, and metastasis (Bhat et al., 2013; Divona et al., 2011; Bonavia et al., 2012; Fan et al., 2005, 2010; Ferris and Grandis, 2007; Gan et al., 2009; Tanaka et al., 2011; Yamamoto et al., 2013). Nonetheless, development of targeted therapeutics for transcription factors such as NF-κB is notoriously challenging. Thus far, NF-κB targeting cancer therapeutics has not been established. In addition, although NF-κB is potentially one of the promising candidates in MES GSCs, we should take into consideration that excessive and prolonged NF-κB inhibition is possibly detrimental because of its suppression in innate immunity (Baud and Karin, 2009; Greten et al., 2007). In this context, this study provides an alternative mode of NF-κB activation that could be exclusive to MES GSCs. The MLK4-driven IKKα/IKBα signaling axis is therefore a therapeutic target for the MES subtypes of GBMs. Moreover, the toxicity of specific MLK4 inhibitors may be modest because MLK4 is not expressed in normal progenitors and normal brain.

In conclusion, this study reveals a critical role of MLK4 in controlling the MES identity in GSCs, and suggests MLK4 as an attractive target for the treatment of aggressive cancers including MES GBM. The poorly characterized serine/threonine kinase MLK4 is abundantly expressed in various cancers (Abi Saab et al., 2012; Martini et al., 2013). Therefore, our data are applicable to other cancer types beyond GBM and provide a rationale for the therapeutic targeting of MLK4 and/or the MLK4/NF-κB complex in a broader context. Given the importance of NF-κB signaling in cancers in general, our findings not only provide a better understanding of the molecular mechanisms underlying the maintenance of stem cell characteristics in GBM but also identify a molecular target for therapeutic intervention. In addition, identification of MLK4-specific inhibitors could potentially create a new paradigm in the discovery and development of molecular targeted therapeutics for cancers including GBM.

**EXPERIMENTAL PROCEDURES**

**Ethics**

All of the work related to human tissues was exempt from requiring consent by the Institutional Review Board (IRB) at University of Alabama at Birmingham (IRB #N151013001 and #N151014002), The Ohio State University (IRB #2005C0075), and M.D. Anderson Cancer Center (IRB #LAB04-0001). All mouse studies were conducted under the approved protocols by Institutional Animal Care and Use Committee and in accordance with NIH guidelines. The unique identity of all patient-derived glioma cell line was confirmed by short tandem repeats analysis as described in Supplemental Experimental Procedures.

**Establishment of GSC Cultures**

HGG patient-derived neurospheres were molecularly characterized as previously described (Bhat et al., 2013; Gu et al., 2013; Joshi et al., 2013; Mao et al., 2013). Freshly resected glioma tumor samples were dissociated and established GSC cells were cultured in defined medium containing DMEM/F12/ Glutamax (Invitrogen) supplemented with B27 (Milltenyi Biotec), heparin (2.5 μg/ml), basic fibroblast growth factor (bFGF; 20 ng/ml), and epidermal growth factor (EGF) (20 ng/ml). Growth factors (bFGF and EGF) were added twice a week.

**Tissue Microarray**

Tissue microarray consisting of three to six representative 0.6-mm cores from formalin-fixed, paraffin-embedded tissue blocks was generated in the Department of Pathology and Laboratory Medicine at The Ohio State University as described previously (Gu et al., 2013; Guvenc et al., 2013; Miyazaki et al., 2013).

**Gaussia NF-κB Promoter Luciferase Assay**

Gaussia luciferase reporter lentiviral vector containing NF-κB binding sites (NF-Gluc-reporter) was provided by Dr. Christian Badr (Badr et al., 2009). After infection with NF-κB-Gluc reporter lentivirus in glioma spheres, 15–μl aliquots of the cell-free conditioned medium were collected. Gluc activity was assayed by adding 20 μM coelenterazine, the Gluc substrate (NanoLight), to the supernatant and measuring photon counts in a 96-well plate FLUOSTar luminesimeter (BMG Labtech) over 10 s.

**Plasmids and Lentiviral Transduction**

Lentiviral vectors expressing non-target shRNA, two shRNA constructs targeting MLK4, shMLK4_1 (clone name: NM_032435.x–1499s1c1) and shMLK4_2 (clone name: NM_032435.x–1413s1c1) used to silence MLK4 expression, were obtained from Sigma. Full-length MLK4 cDNA in pCMV-Entry vector is obtained from Origene and transferred into pLenti-C-Myc-DDK lentiviral overexpression vector with the RapidShuttling kit (Origene). 293FT (Invitrogen) cells were transfected using calcium phosphate (Clontech) for lentivirus production. Lentivirus was harvested at 72 hr after transfection and concentrated 100-fold using LentI-X concentrator (Clontech). Infection of lentivirus was performed according to the manufacturer’s protocol.

**Single-Cell Motility Assay**

Single-cell motility was monitored under guided migration conditions for approximately 16 hr (Gallego-Perez et al., 2012; Irinia and Toner, 2009; Johnson et al., 2009; Petrie et al., 2009). Glioma spheres were dissociated into single cells, plated, and allowed to adhere and spread on micropatterned polydimethylsiloxane surfaces in serum-containing, heparin-free, CO2-independent medium (Invitrogen). Such surfaces were fabricated through a simple replica-molding process from a photolithographically patterned Si master (Gallego-Perez et al., 2012). These substrates were sterilized in 70% ethanol prior to cell seeding. Cell motility was traced via time-lapse microscopy. Images were collected every 10 min, and analyzed using the manual tracker plugin in Fiji.

**Limiting Dilution Neurosphere-Forming Assay**

Limiting dilution assay was performed in 96-well plates as described previously (Flavahan et al., 2013). In brief, dissociated cells from glioma spheres on the 0.5 μg/cm² laminin (Sigma) pre-coated flasks were seeded in 96-well plates containing GSC culture medium (1–50 or 1–100 cells per well). After 7 days for MES 83 and 14 days for MES 267, each well was examined for formation of tumor spheres. Stem cell frequency was calculated using extreme limiting dilution analysis (http://bioinf.wehi.edu.au/software/eldas/).
**Accession Numbers**

The accession numbers for all datasets reported in this paper are GEO: GSE67089 (gene expression arrays) and GSE75224 (gene expression arrays).

**Supplemental Information**

Supplemental Information includes Supplemental Experimental Procedures, five figures, two tables, and two movies and can be found with this article online at [dx.doi.org/10.1016/j.cccel.2016.01.005](http://dx.doi.org/10.1016/j.cccel.2016.01.005).

**Author Contributions**


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Gan, H.K., Lappas, M., Cao, D.X., Cvrljevdic, A., Scott, A.M., and Johns, T.G. (2012). A restricted-in-aid for Scientific Research (B-26293322) from the Japan Society for the Promotion of Science and Takeda Science Foundation (to M.N.); and Career Enhancement Project grant (2P50CA1270011) from the M.D. Anderson Brain Tumor SPORE (to K.B.).


Supplemental Information

Serine/Threonine Kinase MLK4 Determines Mesenchymal Identity in Glioma Stem Cells in an NF-κB-dependent Manner

Supplemental Information for:

Serine/Threonine kinase MLK4 determines Mesenchymal Identity in Glioma Stem Cells in an NF-κB-dependent manner


Inventory of Supplemental Information

Figure S1, Related to Figure 1.

Table S1, Related to Figure 1.

Table S2, Related to Figure 1.

Figure S2, Related to Figure 2.

Figure S3, Related to Figure 4.

Movie S1, Related to Figure 4.

Movie S2, Related to Figure 4.

Figure S4, Related to Figure 5.

Figure S5, Related to Figure 6.

Supplemental Experimental Procedures

Supplemental References
Figure S1, Related to Figure 1. Expression of MLK4 in PN and MES glioma spheres, neural progenitors, and normal astrocytes

(A) Relative *MLK4* mRNA expression levels in PN and MES glioma spheres (18 and 12 samples, respectively), normal astrocytes (Astro), and neural progenitors (NP). Note that *MLK4* is upregulated in MES glioma sphere samples.

(B) Gene expression of MLK family (*MLK1*, 2, 3, and 4), *CD44* (MES marker), *OLIG2* and *SOX2* (PN markers) in PN and MES glioma spheres. Note that *MLK4* is the only MLK gene that is upregulated in MES glioma spheres.

(C) qRT-PCR analysis of mRNA expression of *MLK4* in glioma spheres. Data are means ± SD (n = 3).

(D) Representative immunofluorescence images showing MLK4 expression in PN 84 glioma spheres. MLK4 was labeled in green. Nuclei were stained with DAPI. Scale bar represents 20 μm.

(E) Representative FACS plots showing CD44 and MLK4 in GSC1016.

(F) IF analysis of MLK4 and CD44 expression in MES 83 glioma spheres. MLK4 is uniformly expressed in two neurospheres with different magnified image. Scale bars represent 10 μm and 20 μm, respectively.

(G) Methylation status of *MLK4* promoter region in the six GBM subtypes (Sturm et al., 2012). Red lines indicate hypermethylation, while blue lines indicate hypomethylation. Note that the promoter region for the *MLK4* gene is methylated only in *IDH1* mutant PN GBM tumors.
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Table S2, Related to Figure 1. STR analysis of glioma cell lines used for in vitro and in vivo experiments
The figure shows the effects of shMLK4_2 on MLK4 mRNA levels and cell growth.

**Panel A:** Western blot analysis showing MLK4 protein levels in different samples.

**Panel B:** Western blot analysis showing MLK4 KO and Vector samples.

**Panel C:** Graph showing the relative MLK4 mRNA levels across different treatments.

**Panel D:** Bar graph showing the percentage of apoptosis in shNT and shMLK4_2 treated cells.

**Panel E:** Scatter plots showing the sphere number (10 μm) for PN 528 and MES 83 cell lines under different shRNA treatments.

**Panel F:** Graph illustrating the log fraction of spheres against the number of cells/well, with a linear regression line indicating the stem cell frequency for shNT and shMLK4_2 treatments.
Figure S2, Related to Figure 2. Effects of MLK4 knockdown on cell proliferation and self-renewal activity

(A) Immunoblot (IB) and qRT-PCR analysis showing MLK4 knockdown after transduction with two different shRNAs targeting MLK4 in MES 83 glioma spheres.

(B) Validation of MLK4 knockout (KO) in MES 83 glioma spheres by IB analysis.

(C) Effects of MLK4 knockdown with shMLK_2 on cell proliferation in MES 267 glioma spheres. Cells were plated in 96-well plate at a density of 4,000 cells/well in sphere culture medium, and then analyzed by AlamarBlue staining at the indicated times. The results are expressed in relative fluorescence units (RFU). Data are means ± SD (n = 6). ***p < 0.001.

(D) Apoptotic population in MES 83 glioma spheres expressing shNT, shMLK4_1, or shMLK4_2.

(E) Number of neurospheres (>10 µm) in shNT, shMLK1, shMLK2, or shMLK3 infected PN 528 and MES 83 glioma spheres (20 cells per well of a 96-well plate) grown in sphere culture media for 7 days. Data are means ± SD. ***p < 0.001. Scale bars represent 100 µm.

(F) Effects of shMLK4_2 on sphere forming frequency of MES 267 glioma spheres determined by limiting dilution assays. Varying number of cells (1 to 100) was seeded on each well (18 wells per condition) and allowed to grow for 14 days. Stem cell frequency was calculated by ELDA analysis. ***p < 0.001.
**Figure S3, Related to Figure 4. MLK4 regulates Mesenchymal GSC phenotypes**

(A) Representative images showing actual cells on the patterns. Scale bar represents 50 μm.

(B) qRT-PCR analysis of the expression of mesenchymal representative markers *BCL2A1, TAZ, WT-1*, and *VIM* in MES 83 glioma spheres with vector control or *MLK4* CRISPR/Cas9 (KO). Data are means ± SD (n = 3). **p < 0.01; ***p < 0.001.

(C) GSEA enrichment plot of TCGA PN gene signature in shNT versus shMLK4 of MES 83 glioma spheres. The gene sets for PN GBM signature defined by the TCGA dataset were used for this analysis (Verhaak et al., 2010). The normalized enrichment scores (NES) is shown in the plot.

(D) FACS plots of CD44 expression in MES 267 glioma spheres expressing shNT or shMLK4.

(E) IF analysis of MLK4 expression in GBM xenografts derived from MES 83 and 267 glioma spheres expressing shNT or shMLK4. MLK4 was labeled in red. Nuclei were counterstained with DAPI (blue). Scale bars represent 50 μm.
Movie S1, Related to Figure 4.

Time-lapse imaging of cell motility in MES 83 glioma spheres with non-targeting shRNA control.

Movie S2, Related to Figure 4.

Time-lapse imaging of cell motility in MES 83 glioma spheres with shMLK4_2.
Figure S4, Related to Figure 5. Expression of JNKs, ERKs, and p-p38 in PN and MES glioma spheres

(A) Signal intensity of ERK 1, ERK2, ERK3, ERK4, ERK5, ERK7, JNK1, and JNK2 in PN and MES glioma spheres determined by transcriptome microarray. Six PN and 4 MES glioma sphere samples were used for this analysis.

(B) IB analysis of MLK4 and p-p38 expression in MES 83 glioma spheres expressing shNT or shMLK4. β-Actin as internal control. Note that depletion of MLK4 does not alter p-p38 expression levels.

(C) IB analysis of MLK4, p-IKKα, IKKα, p-p65, and p65 expression in MES 267 GSCs expressing shNT, shMLK4_1, or shMLK4_2. β-Actin as internal control.

(D) IP analysis with anti-DDK antibody followed by IB for anti-HA and anti-His showing the IKKα-MLK4 protein complex in 293T cells. Whole cell lysates as input positive control.

(E) Effects of kinase inactive IKKα (S176A) in PN 528 GSCs on MLK4 (R470C) - mediated NF-Gluc activity. Data are means ± SD (n = 3). *p < 0.05.

(F) Table indicating the higher expression of NF-κB downstream target genes in MES glioma spheres compared to PN glioma spheres.

(G) Analysis of correlation of MLK4 and the 4 genes composing the NF-κB complex in 30 glioma sphere samples. Note that NF-κB1, NF-κB2, RELA, and RELB display statistically significant correlation with MLK4 expression in glioma spheres.

(H) Effects of kinase inactive negative MLK4 (K151A) on cell proliferation in MES 83 glioma spheres. Cells (1,000 cells per well) were plated in 96-well plate in sphere culture media, and then analyzed by AlamarBlue staining at 6 days. Data are means ± SD (n = 6). ***p < 0.001.

(I) IP analysis with anti-DDK antibody followed by IB for anti-HA showing the MLK4 interacts with IKKα which is partially dependent on MLK4 kinase activity in 293T cells. Whole cell lysates as input positive control.
Figure S5, Related to Figure 6. Irradiation in MLK4 deficient MES GSCs increased mouse survival

mouse

(A) IHC analysis of nuclear p-p65 expression in GSC 23 derived mouse xenografts with non-irradiation, irradiation, and shMLK4/irradiation. Scale bar represents 20 µm.

(B) IF analysis of CD44 or VIM expression in GSC 23 derived mouse xenografts with non-irradiation, irradiation, and shMLK4/irradiation. Scale bars represent 20 µm.

(C) Kaplan-Meier survival curves of tumor-burden mice with MES 83 glioma spheres with shMLK4_1 in the presence or absence or irradiation. Irradiation was performed on week 3 after implantation for 4 consecutive days at 2.5 Gy/day.
Supplemental Experimental Procedures

**Xenograft Models and Treatments** For the orthotopic implantation, $1 \times 10^4$ cells for MES 83 and $2.5 \times 10^5$ cells for MES 267 of dissociated glioma sphere cells were stereotactically injected into the left striatum of nude mice. For *in vivo* bioluminescent imaging, luciferase expressing GSCs were established by infection with pCignal lenti-CMV-luc viral particles (SABiosciences) and tumor growth was monitored using IVIS 200 system bioluminescence imaging. Fractionated irradiation (2.5 Gy for 4 consecutive days) to the whole brains of mice with a small field biological irradiator, the XRad 225Cx from Precision x-Ray with Image-guided radiation therapy (IGRT) capability was performed and treatment delivered with the small 1.5 mm cylinder as previously described (Bhat et al., 2013).

**Metabolic Experiment** The extracellular acidification rate (ECAR) was analyzed using the XF-Analyzer (Seahorse Bioscience) and $2 \times 10^4$ were seeded in 96-well Seahorse plates. Cells were equilibrated with XF base media (Seahorse Bioscience part #102353-100) supplemented with 25 mM glucose and 1 mM pyruvate at 37°C for 1 hr in a custom incubator without CO$_2$. ECAR were measured at a baseline and Oligomycin A (Seahorse Stress Kit) were prepared in XF assay medium with final concentration of 4 μM and were injected during the
measurements.

Cell Sorting ALDEFLUOR-positive and ALDEFLUOR-negative subpopulations in MES glioma spheres were separated using ALDEFLUOR kits (StemCell Technologies) described previously (Mao et al., 2013). DEAB was used as an aldehyde dehydrogenase (ALDH) inhibitor to determine the fraction for ALDEFLUOR-positive cells in MES glioma cells.

Gene Expression Microarray Data Analysis. Total RNA was isolated and purified using RNeasy Mini Kit (Qiagen) in shNT or shMLK4 in MES glioma spheres. Samples were processed and hybridized to Affymetrix GeneChip® Human Transcriptome Array (HTA) 2.0, which contain > 6.0 million probes covering the exons of > 65,000 coding and non-coding transcripts. Gene expression microarray data were preprocessed and normalized with Affymetrix Expression Console software (V1.3.1) using RMA sketch method.

Bioinformatics For heatmap and clustering, GSE67089 datasets in the NCBI Gene Expression Omnibus (GEO) were analyzed for gene expression between PN, MES, and other cells. Among 349 kinase genes, the expressions of 56 most highly variable genes (S.D. > 1.2) were extracted and clustered with Cluster 3.0 software, using Pearson Correlation Coefficient as distance measure. The result
was shown with Java Treeview software. Gene set enrichment analysis
(Subramaninan et al., 2005) was used to test the enrichment of
Proneural/Mesenchymal phenotypes related target genes (Verhaak et al., 2010).
NF-κB target gene list was downloaded from http://www.bu.edu/nf-kb/gene-
resources/target-genes/.

**Site Directed Mutagenesis** Site directed mutagenesis is performed using
Quickchange II site-directed mutagenesis kit (Agilent) using following primers:
MLK4 (R470C), Forward: 5’-GCAGCTGGCAGAGTGCGAGATCGACGT-3’,
Reverse: 5’-ACGTCGATCTCGCACCTGCCAGCTGC-3’, MLK4 (K151A), Forward:
5’-GAGGTGGCGGCGGCGGCGCGCAGCCA-3’, Reverse: 5’-TGCGCGCCGCCG-
GCCACGGCCACCTC-3’, IKKα (S176A), Forward: 5’-TGCCAAAGATGTGGATCA-
AGGAGCTCTGTGTACATCTTTTGTG-3’, Reverse: 5’-CCACAAAAGATGTACAC-
AGAGCTCCTTGATCAACATCTTTGGCA-3’.

**EMSA Analysis** EMSA was performed as previously described (Guttridge et al.,
1999). In brief, nuclear extract was prepared and incubated with radio-labeled
probes. Complexes were then resolved on 5% polyacrylamide gels. The gels
were dried and exposed on film for approximately 1 to 3 days.

**In Vitro Kinase Assay** Recombinant human MLK4, IKKα and IKKβ proteins were
purchased from Signalchem. MLK4 kinase activity was measured with a
nonradioactive method using anti-p-Serine antibody (Life technologies).

Reactions with IKKα or IKKβ (100ng) was performed in a total volume of 20 μl with variable amounts of the recombinant kinase MLK4, 100, 200 and 500 ng with 100 μM ATP in kinase buffer (25 mM Tris, pH 7.5, 10 mM MgCl₂, 2 mM EGTA, 1 mM dithiothreitol, and 1 mM sodium orthovandate) at 30°C for 30 min. Reactions were stopped by addition of 10 mM EDTA and stored at -20°C until analysis by immunoblot. Coomassie staining was used to stain IKK α, IKKβ and MLK4 proteins.

**Quantitative RT-PCR** Quantitative Real-Time reverse transcriptase-PCR was performed using SYBR Green dye (Takara) as previously described (Mao et al., 2013). Briefly, ImProm-II Reverse transcription system kit (Promega) was used to synthesize cDNA from 2 μg of total RNA in the presence of random primers (Invitrogen). All reactions were performed in triplicate according to the manufacturer’s recommended thermocycling conditions, and then subjected to melting curve analysis. All oligonucleotide primers were synthesized by Invitrogen. The primer sequences for the transcript analysis are as follows: MLK4, Forward: 5’-CATGAGGAGGCCTTCGTG-3’, Reverse: 5’-CGCCAACCCAAAATCTGTAA-3’; BCL2A1, Forward: 5’-ATGGATAAGGCAAAACGGAG-3’; Reverse: 5’-TGGAGTGTCCTTTTCTGGTCA-3’; WT1, Forward: 5’-CAGCTTGAATGACATGACCTG-3’; Reverse: 5’-TATTCTGTATTGGGCTCCGC-3’; VIM, Forward: 5’-GCCCTTA-
AAGGAACCAATGA-3'; Reverse: 5'-AGCTTCAACGGCAAGTTCT-3'; RUNX2, Forward: 5'-ACAGTAGATGGACCTCGGA-3'; Reverse: 5'-ATACTGGGATGAGGAATGCG-3'; TAZ, Forward: 5'-AGTCCTACGACGTGACCAC-3'; Reverse: 5'-TTCTAGGGTCTTGCCATGT-3'; SNAI1, Forward: 5'-CTCTAGGCCCTGGC-3'; Reverse: 5'-CTCCAGCATTTTACGGACC-3'; Reverse: 5'-GCTGCAAAGCTGGTGTAACACT-3'.

**Immunohistochemistry and Immunofluorescent Staining** For IHC, mice and tumor tissues were perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA). Then, brains were fixed in 4% PFA, paraffin-embedded, and cut into 10 µm (mice) and 4 µm (brain tissues) sections. The slides were incubated with the indicated primary antibodies overnight at 4°C, followed by incubation with an HRP-conjugated secondary antibody for 1 hr at room temperature. Signals were detected using DAB substrate kit (Vector). For IF, cells and tissues were fixed in 4% paraformaldehyde (PFA) in PBS. After blocking and permeabilization with 0.3% Triton X-100 and 1% bovine serum albumin in PBS, cells were probed with following primary antibodies: anti-MLK4 (Bethyl Laboratories), anti-CD44 (Cell Signaling), anti-VIM (DAKO), and anti-OLIG2 (Milipore), p-p65 (Abcam). Alexa 488 and 594 labeled secondary antibodies were used to visualize immunofluorescence signals. Representative images were photographed using
confocal microscopy (Zeiss).

**Immunoblot Analysis** Cells were lysed in RIPA buffer (Sigma) supplemented with proteinase inhibitor and phosphatase inhibitor cocktail (Sigma). Protein samples were quantified using the Bradford assay reagent (Bio-Rad) according to the manufacturer's instructions. Blots were incubated with indicated antibodies for overnight at 4°C. Antibodies against MLK4 (Bethyl Laboratories), HA (Covance), His (Sigma), DDK (Origene), p-p38 (Thr 180/Tyr 182), β-Actin(Cell Signaling), GAPDH (Cell Signaling), and NF-κB sampler kit (Cell Signaling) including p-IKKα/β (Ser 176/180), IKKα, p-NF-κB p65 (Ser 536), p65, p-IκB (Ser 32), and IκB were used. Following wash with TBS-T (TBS containing 0.1% Tween-20), the blot was incubated with horseradish peroxidase-conjugated secondary antibody and the signals were visualized by enhanced chemiluminescence system as described by the manufacturer (GE Healthcare).

**Immunoprecipitation** Cells were harvested and lysed in IP lysis buffer (Pierce) supplemented with protease inhibitors, incubated on ice for 15 min, and cleared by centrifugation at 13,000 rpm at 4°C for 15 min. After preclearing step with protein A/G- agarose beads (upstate), protein lysate (1 mg) was subjected to immunoprecipitation with the agarose-immobilized antibody (2 µg of anti-IKKα (Novus), DDK, or isotype control antibodies) for overnight at 4°C.
Apoptosis Assay Apoptosis assay was performed using the Annexin V antibody (AV) and Propidium Iodide (PI) by following the protocol provided by the apoptosis detection kit (BD Biosciences). Cells without AV or PI were used to detect autofluorescence.

FACS Glioma spheres were dissociated into single cells by Accutase (Sigma) for 5-10 min and stained with CD44-APC antibody (Miltenyi Biotech) according to manufacturer’s instructions. Cells without primary antibody were used for negative control.

Cell proliferation assay (EdU staining). EdU staining was performed using Click-iT™ EdU Alexa Fluor® 488 imaging kit (Invitrogen) according to the manufacturer’s protocol. Dissociated glioma spheres were seeded into wells of laminin pre-coated 8 well chamber slides. The next day, EdU was directly added into the culture media at a final concentration of 10 µM and incubated for 2 hr, and cells were fixed with 4% paraformaldehyde in PBS for 15 min, followed by permeabilization with 0.5% Triton X-100 for 20 min. Nuclei were counterstained with Hoechst 33342.

Small interfering RNA(siRNA) Knockdown of human IKKα was performed by
transfection of Dharmacon SMARTpool ON-TARGETplus CHUK siRNA
(L-003473-00-0005-Dharmacon) or ON-TARGETplus Non-targeting siRNA#1
(D-001810-01-20-Dharmacon) using a Lipofectamine RNAiMAX reagent.

**CRISPR-Cas9 mediated gene knockout.** Cells were seeded at 4X10^5 cells/well
in 6-well plate and transfected 2 µg *MLK4* CRISPR/Cas9 KO plasmid (Santa Cruz
Biotechnology) or control CRISPR/Cas9 KO plasmid into MES 83 GSCs with
Lipofectamine 2000. After 48 hr transfection, GFP positive cells were isolated by
sorting on a FACS-Aria cell sorter (Becton Dickinson) and seeded as single cells
in the well of 96-well plate. The cells from each well were amplified and selected
by IB analysis.

**Osteogenic differentiation** MES glioma spheres were seeded on collagen
(Corning) pre-coated 6-well plate at 5 X 10^4 in sphere culture medium for 24 hr.
To induce osteogenic differentiation, the media were then replaced with
osteogenic differentiation media (Lonza). At indicated time points, cells were
harvested for qRT-PCR.

**Cell Line STR Analysis**

Services were provided by University of Arizona Genetics Core, Arizona
Research Laboratories, Division of Biotechnology, http://uagc.arl.arizona.edu/.
Genomic DNA was purified from individual cell lines and shipped on dry ice to Core Facility. PCR was performed and products were separated by capillary electrophoresis using an AB 3730 DNA Analyzer. Applied Biosystems Internal Size Standard (ISS) GeneScan500-LIZ (PN:4322682) was used to standardize allele size calls for each sample. The PCR product was diluted 1:50 with sterile H₂O and prepared with POP7 Polymer (PN:4335615) per the manufacturer's instructions (Applied Biosystems). Samples were run on a 36 cm capillary array-Applied Biosystems (PN:4331247). For data analysis, electropherograms were analyzed from the .fsa files and allelic values assigned using Soft Genetics, Gene Marker Software Version 1.85. Alleles were matched to STR Profile recorded with DMSZ (when reference profile is available) thus evaluating whether the profiles from the samples submitted match cell line profiles in these databases. DMSZ contains reference profiles from their cell collection, JCRB and ATCC. DMSZ uses an EV Value to rank degree of matching between the questioned profile and all in the database. This EV value is a method of ranking the results and is not sole statistical metric by which we evaluate a match. We use the ANSI (ANSI/ATCC ASN-0002-2011-Authentication of Human Cell Lines: Standardization of STR Profiling) standard of a minimum 80% match threshold to indicate a shared genetic history.

Percent match equation-

% match between two cell lines = (# of shared alleles in both STR profiles) /
(total # number of alleles in the questioned profile)

Homozygous alleles are counted as one allele.

Cell lines with a percent match between 55%-80% require further genetic profiling to authenticate relatedness. Cell lines with <55% matching between questioned and reference profiles are not considered to share a genetic origin.
Supplemental References