Malignant astrocytomas are the most common and lethal primary human brain tumor, exhibiting multiple molecular aberrations. We report that loss of the transcription factor GATA4, a negative regulator of normal astrocyte proliferation, is a driver in glioma formation and fulfills the hallmarks of a tumor suppressor gene (TSG). Although GATA4 was expressed in normal brain, loss of GATA4 was observed in 94/163 GBM operative samples and was a negative survival prognostic marker. GATA4 loss occurred through promoter hypermethylation or novel somatic mutations. Loss of GATA4 in normal human astrocytes promoted high-grade astrocytoma formation, in cooperation with other relevant genetic alterations such as activated Ras or loss of TP53. Loss of GATA4 with activated Ras in normal astrocytes promoted a progenitor-like phenotype, formation of neurospheres, and the ability to differentiate into astrocytes, neurons, and oligodendrocytes. Re-expression of GATA4 in human GBM cell lines, primary cultures, and brain tumor–initiating cells suppressed tumor growth in vitro and in vivo through direct activation of the cell cycle inhibitor P21\(^{33,33}\),
GATA4 represses astrocytoma formation | Agnihotri et al.

The GATA family of transcription factors consists of six members, with two conserved zinc finger domains that recognize the consensus DNA binding motif of (A/T)/GATA/(A/G) (Molkentin, 2000). They regulate biological functions, including organogenesis, differentiation, proliferation, and apoptosis (Kuo et al., 1997; Charron et al., 1999; Koutsourakis et al., 1999; Holtzinger and Evans, 2005; Kobayashi et al., 2006; Watt et al., 2007), but their roles in the normal and transformed human central nervous system (CNS) remain in large part unknown.

Our interests have focused on GATA4 and GATA6 because we observed no significant alterations in GATA5 expression between normal brain and HGAs. We have previously reported on the expression profile of GATA6 in the CNS (Kamnasaran and Guha, 2005) and recently reported GATA4 expression in normal embryonic and adult mouse and human astrocytes, in which it functions as an inhibitor of proliferation and inducer of apoptosis (Agnihotri et al., 2009). GATA4 knockout mice are embryonic lethal (embryonic day [E] 7.5–8.5) as a result of cardiac defects (Kuo et al., 1997; Pehlivan et al., 1999; Reamon-Buettner et al., 2007), and GATA4 mutations cause Holt-Oram syndrome and congenital heart defects. GATA4 is frequently silenced in lung, colon, prostate, ovarian, and breast cancer (Akiyama et al., 2003; Guo et al., 2004, 2006; Caslini et al., 2006; Hua et al., 2009), but its exact role in cancer biology and the mechanisms by which it operates are poorly understood. Given the role of GATA4 in regulating astrocyte proliferation and the observed loss of GATA4 in several human cancers, in this study we demonstrate GATA4 to be a novel tumor suppressor in GBM, and we identify novel mechanisms of tumor suppression regulated by GATA4.
specimens (Fig. 1, C and D). The specificity of the anti-GATA4 antibody was tested using confirmed GBM tissue that did not express GATA4 at the transcript or protein level (Fig. S1 A). Tissue microarray analysis of primary GBM linked with survival data (Cloughesy et al., 2008; Guo et al., 2009; n = 43, primary GBM) demonstrated poorer overall survival in patients with loss of GATA4 expression, compared with patients with GATA4 expression (Fig. 1 E; P = 0.01, Log-rank test). In summary, GATA4 loss is observed in a majority of human GBM lines and operative samples.

Promoter methylation and somatic mutations silence GATA4 expression

To establish the etiology of GATA4 loss in human GBMs, we first determined whether there was epigenetic silencing by hypermethylation of the GATA4 promoter, as demonstrated in lung and colon cancer (Akiyama et al., 2003; Guo et al., 2004; Hellebrekers et al., 2009). The 1,200-bp GATA4 proximal

| RESULTS |

Loss of GATA4 is observed in a majority of GBMs

We established nontransformed primary astrocyte cultures from normal control mice (newborn normal mouse astrocyte [NMA] postnatal day [P] 0 and 3-mo-old NMA P3), both of which expressed Gata4 (Fig. 1 A). Cultures derived from our newborn GFAPV12Ha-Ras glioma-prone mice, RasB8 P0, expressed Gata4, whereas astrocytoma cultures from 3-mo-old tumor-bearing GFAPV12Ha-Ras mice, RasB8 P3, had complete loss of Gata4 (Fig. 1 A). There was no variation in Gata5 expression between NMAs and mouse astrocytoma cells (Fig. 1 A). Complete loss of GATA4 but not GATA5 was observed in 10/11 human GBM lines compared with normal human astrocytes (NHAs) and those which were immortalized but not transformed with hTERT (NHA + hTERT; Fig. 1 B).

We then examined the status of GATA4 expression in patient tumor specimens. GATA4 protein expression was lost in 15/29 (~50%) LGAs and 94/163 (~60%) human GBM specimens (Fig. 1, C and D). The specificity of the anti-GATA4 antibody was tested using confirmed GBM tissue that did not express GATA4 at the transcript or protein level (Fig.S1 A). Tissue microarray analysis of primary GBM linked with survival data (Cloughesy et al., 2008; Guo et al., 2009; n = 43, primary GBM) demonstrated poorer overall survival in patients with loss of GATA4 expression, compared with patients with GATA4 expression (Fig. 1 E; P = 0.01, Log-rank test). In summary, GATA4 loss is observed in a majority of human GBM lines and operative samples.
promoter has two dense CpG islands, which are sites of increased methylation (Fig. 2 A). Methylation-specific (MSP) PCR, which is able to differentiate unmethylated and methylated products of the GATA4 promoter, demonstrated that the methylated product was amplified from all human GBM lines lacking GATA4 expression (Fig. 2 A). In contrast, normal human brain (NHB) and NHA had predominantly the nonmethylated product (Fig. 2 A). Treatment of several GBM lines with 5-aza-2’-deoxycytidine, an inhibitor of DNA methyltransferases, restored GATA4 protein expression (Fig. 2 B). We next performed MSP-PCR on bisulfite-treated DNA isolated from GBM operative samples lacking GATA4 at the transcript level (Fig. S1 B). 13/20 of these GATA4-negative samples had a methylated product amplify using MSP-PCR (Fig. S1 C). To further validate GATA4 promoter methylation, bisulfite sequencing, which has increased sensitivity compared with MSP-PCR, was used to analyze 36 CpG islands in the GATA4 promoter gene (Fig. S1 G). Primers used were specific to the transgene construct and not endogenous GATA4 (Fig. S1 G).

To determine alternate methods of gene silencing, we subjected the 7/20 GBMs in which the GATA4 transcript was absent, but without promoter methylation, to exon sequencing. We identified three novel insertion/deletion (indel) mutations that resulted in frameshifts. These mutations were somatic and not germline because the mutations were not found in matched patient blood samples (Fig. 2 E). The mutations were located in the Zinc finger domains as well as the C terminus of GATA4 (Fig. 2 F). Site-directed mutagenesis corresponding to these three mutations on WT GATA4 cDNA resulted in loss of GATA4 function, as they could not activate a P21-CIP-Lux construct (Fig. 2 F; P = 0.001). Loss of heterozygosity (LOH) accompanied these mutations, as determined by single nucleotide polymorphism (SNP) markers flanking the mutations (Fig. S2, A–C). Thus, GATA4 silencing in GBMs is primarily through promoter methylation and somatic mutations with accompanying LOH.

**Loss of GATA4 promotes HGA formation in vitro and in vivo**

Because GATA4 was lost in the mouse GFAP-V12Ha-Ras HGAs and majority of human GBM lines and specimens, we postulated that GATA4 serves as a TSG in GBMs. First, loss of Gata4 in RasB8 P0 nontransformed astrocytes promoted proliferation and transformation in vitro (Fig. S3, A–C). The transformation synergy was not restricted to activated Ras because NHA + hTERT immortalized astrocytes expressing E6/E7 oncoproteins (to inhibit both TP53 and RB pathways) were also transformed in vitro by shRNA-mediated GATA4 knockdown (Fig. S3, A–C). To further test our postulate, we generated two stable GATA4 shRNA NHA + hTERT cells (nontransformed astrocytes expressing GAF and no NESTIN), which resulted in increased proliferation but not transformation as measured by soft agar assay (Fig. S3 D and not depicted). However, in combination with V12Ha-Ras, to mimic aberrantly expressed Ras in GBM through NF1 loss or aberrant receptor activation (TCGA network, 2008), we observed that loss of GATA4 promoted not only proliferation but also growth in soft agar (Fig. 3 A and Fig. S3 E, P = 0.0001). This tumorigenic phenotype of the NHA-V12Ha-Ras-GATA4 shRNA1 cells was rescued by expressing GATA4 cDNA lacking part of the 3’UTR to which the shRNA1 was directed against (Fig. 3 A and Fig. S3 E, P = 0.001).

NHA-V12Ha-Ras-GATA4 shRNA1 cells were then injected intracranially into NOD-SCID mice (n = 5) with NHA-V12Ha-Ras-GATA4 scrambled shRNA cells as controls. The control cell–injected mice survived without any tumors when evaluated at 60 d, which is consistent with the nontumorigenic phenotype previously reported for NHA-V12Ha-Ras cells (Sonoda et al., 2001; Fig. 3 B, top left). In contrast, mice injected with NHA-V12Ha-Ras-GATA4 shRNA1 cells formed tumors (5/5) and died from HGAs by 31 ± 7 d (Fig. 3 B, all except top left). The HGAs had elevated Ki-67 index and cyclin D1 (CCND1) expression (Figs. S4 A and S3 E) and reduced expression of the cell cycle inhibitor P21(CIP1) (Fig. 3 B). These tumors did not express the differentiated astrocyte marker GFAP but had increased levels of NESTIN, a marker for neural progenitor cells which is highly expressed in human GBMs (Fig. 3 B, middle row, left and middle). Primitive neuroectodermal tumors (PNETs) also lack GFAP expression but express high NESTIN levels, but the tumors were not PNET based on tumor morphology and lack of the PNET/neuronal markers NeuN and Synaptophysin (Fig. S3 F). GFAP immunohistochemistry (IHC) analysis of 47 GBMs (serial sections of GBMs stained for GATA4 in Fig. 1 D) demonstrated that loss of GATA4 was associated with loss of GFAP (Fig. S5, A and B, P = 0.0087, Fisher’s exact test). In addition, GATA4 protein was enriched in GFAP promoter by chromatin immunoprecipitation (ChIP) analysis (Fig. S5 C).
other relevant GBM genetic alterations promotes HGA formation with dedifferentiation.

Real-time quantitative (q) RT-PCR analysis of the transformed NHA-V12Ha–Ras–GATA4 shRNA1 cells demonstrated loss of GATA4 and GFAP as noted in the previous paragraph (Fig. S3 G). In addition, these cells had increased levels of markers such as NESTIN, SOX2, and Musashi1 (MSI1), which are known to be associated with brain tumor cell markers. Our observation of GATA4 loss with activated Ras up-regulated putative brain tumor stem cell markers led us to hypothesize that these cells acquired progenitor-like characteristics. In direct support of this, NHA–Ras cells with knockdown of GATA4, but not NHA–Ras control cells, were able to form neurospheres (Fig. 3 D, P < 0.0001) in neural progenitor growth conditions and acquired the ability to differentiate into the three major CNS cell lineages: astrocytes, neurons, and oligodendrocytes, based on marker expression, when grown in differentiation conditions (Fig. 3 E). In summary, loss of GATA4 in cooperation with

Figure 3. Loss of GATA4 promotes transformation. (A) NHAs expressing activated Ras were transfected with control or GATA4–specific shRNA, with or without an shRNA-resistant GATA4 construct (GATA4 rescue). Left, GATA4 expression measured by Western blotting. Right, BrdU incorporation measured on indicated days. *, P = 0.02. (B) NOD-SCID mice were subjected to intracranial xenograft with cells described in A. Brains were analyzed by IHC. (top row, left and middle) 500 μm; (middle row, left) 50 μm; (all others) 25 μm. (C) Expression of indicated surface markers was quantified from staining shown in B from five mice. Mean and SEM are represented. (D) Cells described in A were cultured under conditions promoting differentiation into astrocytes (indicated by GFAP expression), neurons (indicated by TUJ1 expression), and oligodendrocytes (indicated by O4 expression). Cells were stained with the indicated antibodies, DAPI, and nuclear stain. Bar, 16 μm. A, D, and E were performed in triplicate with mean and SEM reported where appropriate. B and C are representative stains from five mice.

GATA4 expression in the four GBM cells tested without endogenous GATA4 expression resulted in reduced cyclin
D1 expression and increased levels of the tumor suppressors P21CIP1 and P15INK4B (Fig. 4 B). The induction of cell cycle inhibitors and cyclins by other GATA transcription factors has been previously demonstrated by us and others (Perlman et al., 1998; Setogawa et al., 2006; Agnihotri et al., 2009); however, our observation of the induction of P21CIP1 or attenuation of CCND1 by GATA4 has not been reported.

We next generated a stable U87 GBM cell line expressing GATA4 under a doxycycline-inducible promoter (Fig. 4 C). Induction of GATA4 by doxycycline attenuated levels of cyclin D1 with induction of P21CIP1 (Fig. 4 C). Decreased expression of CCND1 by GATA4 was not at the transcriptional level but through increased ubiquitination by the 26s proteosome (Fig. S4D), as it was reversed by the proteosomal inhibitor MG132. Furthermore, to determine direct ubiquitination of CCND1, U87 cells were transfected with an expression plasmid expressing HA-tagged ubiquitin (HA-UB). Only under GATA4-expressing conditions was HA-UB directly incorporated into CCND1 (Fig. S4 E). The change in CCND1 and P21CIP1 protein levels corresponded with reduced cell proliferation and a significant increase of cells in the G1 phase of cell cycle (Fig. 4 D and Fig. S4 C). To determine whether direct loss of CCND1 and/or overexpression of P21CIP1 resulted in the same phenotype as exogenous GATA4 expression, these alterations were introduced in T98G cells. Similar to exogenous GATA4, reduction of CCND1 or induction of P21CIP1 led to a significant decrease in proliferation, which was comparable to expression of GATA4 alone (Fig. 5, A and B; ANOVA, P < 0.01). However, only GATA4-expressing T98G cells were sensitized to cell death when exposed to temozolomide, a standard chemotherapeutic in GBM treatment (Fig. 5 C).

**Figure 4.** Re-expression of GATA4 suppresses cell proliferation and in vivo tumor growth. (A) Transient expression of GATA4 under a constitutive active CMV promoter or empty vector control (EV) decreases proliferation in several GBM cell lines of varying genetic backgrounds. *, P < 0.01 (B) Transient expression of GATA4 leads to attenuation of cyclin D1 with increased levels of P21CIP1 and P15INK4B. (C and D) Doxycycline was added to U87 GBM cell line expressing GATA4 under a doxycycline-inducible promoter. (C) At indicated time points, GATA4, P21CIP1, and CCND1 were measured by Western blotting. (D) At the indicated times, cells in culture were counted. *, P = 0.001. (E) Survival curve of NOD-SCID mice were injected with intracranial xenografts of U87 cells with GATA4 under doxycycline (DOX)-inducible promoter. Mice were continually given doxycycline in drinking water (GATA4 on, n = 10) or no doxycycline (GATA4 off, n = 10). (F) Immunohistochemical analysis of protein markers from tumors of mice not given dox (no GATA4) versus mice given dox (GATA4). Bars: (first H&E column) 500 µm; (second [magnified] H&E column) 50 µm; (GATA4, P21-CIP1, and ki67 columns) 25 µm. (G) Quantification of protein staining from F. n = 5 mice. *, P < 0.01. Mean and SEM are shown. A and D were performed in triplicate with mean and SEM reported.
Intracranial injection of $2.5 \times 10^5$ inducible U87 GATA4 cells in NOD-SCID mice was undertaken to determine in vivo growth effects of GATA4. All 10 of the control mice (− doxycycline) died of HGA's at 32.8 ± 6.2 d (Fig. 4 E). In comparison, the 10 mice receiving doxycycline in their drinking water were all viable and symptom free when sacrificed at 120 d. Necropsy analysis demonstrated no brain tumors in seven mice, whereas three mice had small tumor growth with reduced proliferation and increased levels of GATA4 and P21<sup>CIP1</sup> (Fig. 4, F and G).

We investigated the status of GATA4 in GBM brain tumor initiation cells (BTICs), which are postulated to be responsible for tumor initiation and maintenance and resistance to therapy (Alcantara Llaguno et al., 2009). These BTICs are able to self-renew, proliferate, and give rise to different lineages (Singh et al., 2004; Stiles and Rowitch, 2008). We screened two GBM BTIC lines generated from operative tissue as previously described (Pollard et al., 2009). G179 had significant reduction of GATA4 at the RNA and protein level compared with NHA and HF-240, a nontransformed neural stem cell line (Fig. 5 D). We next generated a stable G179 GATA4-expressing line (Fig. 5 D) and examined the effect on proliferation and sensitivity to temozolomide. G179 cells expressing GATA4 had significant reduction in proliferation by day 5 compared with G179 empty vector control lines cells grown in stem cell media conditions (Fig. 5 E). G179 GATA4-expressing lines were also sensitized to cell death when exposed to temozolomide (Fig. 5 F; P = 0.016). In addition to reduced proliferation, G179 GATA4-expressing cells had significant reduction of several candidate brain tumor stem cell markers: NESTIN, SOX2, and MSI-1 (Fig. 5 E). No changes in CD133 expression were observed. Lastly, we observed increased differentiation potential of G179 GATA4-expressing lines under differentiation conditions (stem cell media + 1% FBS) with significant increase of GFAP (astrocyte lineages) and TUBB3 (neuronal lineages) compared with controls (Fig. 5 F). Loss of GATA4 in our nontransformed HF-240 line was insufficient to increase proliferation (Fig. 5 E) but did impair NESTIN, GFAP, and TUBB3 expression during differentiation conditions (Fig. 5, G and H).

To complement our findings in GBMs, GATA4 loss was observed in several medulloblastoma and non-CNS tumor cell lines such as breast, lung, and prostate (Fig. S6, A and B). Stable expression of GATA4 in PC3 (prostate), A549 (lung), and MD-231 (breast) resulted in decreased proliferation and reduced anchorage-independent growth (Fig. S6, C–F).

In summary, transient or stable induction of GATA4 leads to diminished cellular proliferation and reduced transformation in vitro and in vivo.

![Figure 5.](image)

**Figure 5.** GATA4 negatively regulates GBM growth. (A) Western blot demonstrating protein levels of GATA4, P21-CIP, CCND1, and APNG in T98G cells under varying treatments. MGMT was used as a loading control. (B) BrdU assay of T98G cells by modulation of GATA4, P21-CIP, or CCND1. * P < 0.01 (ANOVA). (C) GATA4-mediated sensitivity to 100 µm temozolomide in vitro (T98G cells; * P = 0.001, ANOVA). Cell death under non-temozolomide was statistically insignificant (P > 0.05, ANOVA). (D, Top) qRT-PCR GATA4 expression in brain tumor initiating cells G144, G179 compared with NHA's, normal neural stem cells (NSC), and the embryonic stem cell line (H9). (D, Bottom) Western blot assessing GATA4 protein status in the lines mentioned for D, Top. * P > 0.05. (E) qRT-PCR of neural stem cell markers in G179 cells. Cells were grown in neural stem cell media. *, P < 0.01. (F) qRT-PCR of neural stem cell markers, GFAP (astrocytic), NKX2.2 (oligodendrocytic), and TUBB3 (neuronal). *, P < 0.01 in G179 cells. Cells were placed in differentiation conditions (neural stem cell media + 1% fetal bovine serum) for 12 d before RNA extraction. All experiments were performed in triplicate with mean and SEM reported.

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GATA4 mediates its tumor suppressive effect through P21CIP1

GATA4-mediated decreased proliferation and increased the percentage of cells in G1, strongly suggesting a role in regulating cell cycle. GATA4 expression in several GBM cell lines consistently increased P21CIP1 protein levels (Fig. 4 B). We sought to determine whether P21CIP1 was a direct target of GATA4 as P21CIP1 transcript levels increased with GATA4 (Fig. S7 A). We focused on P21CIP1 induction by GATA4 in GBMs because our prior studies had already demonstrated that GATA4 induces P15INK4B in normal human and mouse astrocytes (Agnihotri et al., 2009). The P21CIP1 promoter has several GATA binding elements, two of which were enriched by ChIP analysis (Fig. 6 A). Furthermore, transactivation luciferase assays with a −2.6-kb P21CIP1:Luc construct induced luciferase expression in the presence of GATA4 (Fig. 6 B). Site-directed mutagenesis to eliminate the GATA elements in the two promoter luciferase constructs (MS1/MS2 for P21CIP1) ablated the ability of GATA4 to drive luciferase expression (Fig. 6 B). To determine if P21CIP1 is a key downstream effector of GATA4, we transiently transfected U87 cells with a GATA4 cDNA expression construct and two P21CIP1 siRNAs (Fig. 6 C). Decreased P21CIP1 expression in these U87 cells significantly blocked, but did not eliminate, the ability of GATA4 to reduce proliferation (Fig. 6 D). Loss of P21CIP1 by itself further increases proliferation in U87 cells. *P < 0.001. (C and D) Silencing of P21CIP1 with overexpression of GATA4 rescues the antiproliferative effect of GATA4 but not to parental levels, *P < 0.001; **P = 0.002. Loss of P21CIP1 by itself further increases proliferation in U87 cells. **P < 0.001. (E) Western blot demonstrating overexpression of GATA4, loss of P53, and changes to P21CIP1 levels. Doxorubicin was used to activate TP53. (F) P21CIP1 luciferase assay in U373 cells (mutant TP53), and GATA4 and WT TP53 lead to a synergistic increase of luciferase expression. *P = 0.001, lane 5 compared with lanes 2 and 3. Experiments were performed in triplicate with mean and SEM reported.
GATA4 sensitizes GBM cells to temozolomide independent of O-6-methylguanine-DNA-methyltransferase (MGMT)
The negative survival associated with GATA4 loss in GBM patients led us to hypothesize that GATA4 loss may be involved in chemotherapeutic resistance, such as temozolomide. Transient expression of GATA4 decreased viability of U87 and T98G GBM cells to temozolomide in a dose-dependent manner (Fig. 7, A and B). The reduced cell viability correlated with increased apoptosis as cells subjected to GATA4 and temozolomide had a significant increase of the sub-G1 population and increased levels of cleaved PARP (Fig. 7 C; and Fig. S7, E and F). We next used established and primary GBM cell lines (Sarkaria et al., 2006) to evaluate whether the GATA4-mediated sensitization was dependent on TP53 status and MGMT, the DNA repair enzyme which is most often implicated in temozolomide resistance (Hegi et al., 2004, 2005). T98G plus GBM6 (mutant TP53 and MGMT positive) and U87 plus GBM8 (wtTP53, MGMT negative) cells demonstrated similar sensitization to temozolomide resulting from GATA4 expression (Fig. 7 B). A surprising finding was that levels of MGMT were not altered (Fig. 7 C).

Excluding O6 guanine methylation, which is repaired by MGMT, temozolomide alkylates DNA at the N7 position of guanine and the N3 position of adenine (Denny et al., 1994), lesions which are repaired by alkylpurine-DNA-N-glycosylase (APNG; Hang et al., 1997). Transient transfection of GATA4 reduced levels of APNG (Fig. S7 G) in all established and primary GBM lines tested. Furthermore, T98G and GBM6 lines stably expressing GATA4 decreased APNG levels with no reduction of MGMT expression (Fig. 7 D).

To directly test the role of APNG in temozolomide resistance, we treated T98G cells with MGMT and APNG siRNA individually and in a double knockdown (Fig. 7 E). Loss of APNG or MGMT had no effect on cell viability under normal conditions (unpublished data; P > 0.05, ANOVA). Under temozolomide conditions, compared with control cells, loss of APNG sensitized cells to cell death in a dose-dependent manner (Fig. 7 F; P < 0.0001). Co-loss of both MGMT and APNG resulted in enhanced sensitization to temozolomide-induced cell death (Fig. 6 F). APNG siRNA2 and MGMT siRNA2 had similar results (unpublished data). Interestingly, GATA4 regulation of APNG is not at the transcript level, suggesting an alternative method of GATA4 regulating APNG (Fig. S7 H). Together, these results suggest that GATA4 sensitization of GBM cells to temozolomide is mediated through loss of APNG.
DISCUSSION

The identification and validation of GATA6 as a TSG in human GBMs (Kamnasaran et al., 2007) led to our interest in other GATA transcription factors, namely the GATA4,5,6 subfamily. Our expression profiling of GATA4, but not GATA5, demonstrated loss of expression in a majority of human GBM lines and specimens. Recent genome-wide studies have also reported reduced expression of GATA4 in 70% of samples analyzed, with reduced copy number and copy-neutral LOH in 5% of GBM samples (Cancer Genome Atlas Research Network, 2008; Parsons et al., 2008). In additional support of our thesis, we demonstrated ubiquitous expression of GATA4 in normal embryonic and adult astrocytes, where it functions as a negative regulator of astrocyte growth (Agnihotri et al., 2009). Direct evidence comes from data presented in this manuscript where GATA4 fulfills the hallmarks of a TSG in cancer development (Haber and Harlow, 1997), specifically human astrocytomas. GATA4 expression was lost in human GBMs through promoter methylation and somatic mutations associated with LOH. Interestingly we observed no transcript associated with these mutations. Given the fact that these mutations occur in exons near intron/exon boundaries, it is highly suggestive that these mutant transcripts are targeted for destruction by the mRNA nonsense-mediated decay pathway (Maquat, 2004). Loss of GATA4 in human and mouse astrocytes promoted HGA formation in vivo, in conjunction with other genetic alterations. Reexpression of GATA4 suppressed astrocytoma growth in vivo. It is of interest that we observed a higher frequency of GATA4 loss in LGAs (>50%) compared with our result for GATA6 (~20%). This, along with the present findings that GATA4 can negatively regulate brain tumor–initiating cells, suggests that GATA4 loss is an earlier event in astrocytoma genesis.

Our study is the first mechanistic investigation of the tumor-suppressive actions of GATA4, whose loss has been documented in other human cancers (Cai et al., 2009; Hellebrekers et al., 2009; Hua et al., 2009). We identified P21CIP1 as a direct transcriptional target of GATA4, with attenuation of the potent oncogene cyclin D1 through ubiquitination. This is of great interest, as GATA transcription factors have never been implicated in regulation of ubiquitination, a biological process which is poorly characterized in GBM. Our data demonstrate that induction of P21CIP1 is not entirely dependent on TP53, but additional pathways are likely to be used by GATA4 to induce P21CIP1 and inhibit cell cycle and astrocytoma proliferation. In addition, the fact that removal of P21CIP1 did not eliminate all of the antiproliferative effects of GATA4 suggests that mechanisms outside of P15INK4B and P21CIP1 exist for GATA4-mediated growth suppression. Identification of these target genes are of critical interest. In a recent study, four subtypes of primary GBM based on gene expression profiling were identified, with the proneural subtype having the greatest decrease of GATA4 (Verhaak et al., 2010).

Loss of GATA4 also correlated with loss of GFAP at the protein level, which is a surrogate marker for GBM tumor differentiation. Interestingly, GATA4 was found to be associated with the C1.1 region of the GFAP promoter. This is of high interest, as this region contains a highly conserved GATA element among several mammalian species (Lee et al., 2008). Although this may imply GFAP regulation by GATA4, the co-loss may also arise as a result of the fact that GATA4 and GFAP are both targeted for epigenetic silencing (Reestrepo et al., 2011; this paper). GATA4’s role in embryonic stem cell and endoderm differentiation has been well studied. The ability of GATA4 reexpression in the BTIC line G179 illustrated that GATA4 has a prodifferentiation effect in GBM BTIC lines with decreased proliferations. This is further complemented by the fact that NHAs expressing activated RAS and loss of GATA4 acquired progenitor-like phenotypes and that loss of GATA4 in the nontransformed neural stem cell (HF-240) reduces astrocytic and neuronal expression markers GFAP and TUBB3 at the RNA level. Although HF-240 cells express GATA4, its expression is lower compared with differentiated astrocytes (NHAs). Therefore, the exact role of GATA4 and the relevance of expression level remain to be elucidated in neural stem cells.

We also observed that GATA4 loss is a negative survival factor in GBM patients treated with current standardized care involving surgery, radiation, and temozolomide. Our results demonstrate sensitization of GBM cells by expression of GATA4, which is independent of TP53 and MGMT status of the cells. Our observation is that APNG, which is involved in repairing another site of DNA methylation by temozolomide, is of potential therapeutic interest in perhaps modulating GBM sensitivity to temozolomide and other alkylating agents independent of current interests in MGMT. This last point is of unusual interest, as it demonstrates how DNA repair enzymes, caretakers of DNA damage in normal cells, can be a major hurdle and contribute to chemotherapeutic resistance in cancer cells, not only limited to GBM. This is of clinical importance, as it provides a potential therapeutic target and offers an alternative explanation as to why some MGMT-positive patients are still responsive to temozolomide or why some MGMT-negative patients are resistant to temozolomide. Although we were unable to determine how GATA4 attenuates APNG, we hypothesize that it may be at the protein level, such as in ubiquitination by activation of unknown GATA4-regulated E3 ligases.

Furthermore, GATA4 loss in medulloblastoma cell lines and various other cancer cell lines at the protein level (Fig. S6, A and B) may suggest that GATA4’s effects are not restricted to just GBM. In direct support of this, stable restoration of GATA4 in lung, prostate, and breast cancer cell lines suppressed proliferation. These findings suggest that GATA4 exerts TSG properties in other cancers, in addition to our findings in GBM, and that the role of GATA4 in these cancers where it is silenced should be further investigated.

In summary, we have identified and validated a novel human TSG in human astrocytomas, with work initiated from well characterized mouse models. These replenishable mouse models, with limited genetic variability, not only facilitate our understanding of and interactions with known genetic alterations in human cancer but also serve to elucidate novel genetic
alterations with gene discovery strategies such as gene trap screens. Careful validation in human samples and mechanistic studies are required and collectively augment the findings from large-scale human cancer initiatives.

**MATERIALS AND METHODS**

**Tumour specimens.** NBH, low grade, and GBM operative samples were obtained from the IREB-approved Nervous System Tumor Bank at University Health Network (UHN; http://www.brain tumour bank.ca/index.html), Toronto. Additional GBM-containing tissue microarrays were obtained from the MD Anderson Cancer Center (G. Fuller and K. Aldape) and University of California, Los Angeles (P. Mischel).

**Xenograft studies.** Mice were maintained in accordance with UHN institutional animal protocols. Stereotactic guided intracranial injections in NOD-SCID mice were performed by injecting 250,000 cells of NHA-Ras + shRNA control, NHA-Ras + GATA4 shRNA1, U87-TRE-GATA into the frontal cortex (coordinates were X = −1.0, Y = 1.5, Z = 2.4, with Bregma serving as the 0 point for X and Y).

**Cell lines and primary cultures.** U87 and T98G cell lines were obtained from American Type Culture Collection, NHA and NHA + TETR cells were obtained from R.O. Peper (University of California, San Francisco, CA) and U373 and D423 cells were obtained from D Biguer (Duke University, Durham, NC). The cells were grown in DME supplemented with 10% FBS at 37°C in a 95% air/5% CO2 atmosphere. Primary mouse astrocytes were established from newborn or adult control CD1 mice or the RasB8 mouse model as previously described (Luo et al., 2000; Kamnasaran et al., 2007). The purity of mouse astrocytes was >95%, as determined by immunofluorescence cytochemistry assay with anti-glial fibrillary acidic protein antibody (astrocyte-specific marker). GBM6 and GBM8 xenograft lines were established from serially passaged mice xenograft models as previously described (Sarkaria et al., 2006).

**Mutation sequencing.** GATA4 exons and neighboring intronic regions were amplified using PCR with Hifi fidelity Taq (Invitrogen) and sequenced at The Centre for Applied Genomics Sequencing Facility (Hospital for Sick Children’s Research Institute, Toronto, Ontario, Canada). Mutations identified were validated in triplicate by three separate PCR reactions and sequencing runs. Mutations into WT GATA4 cDNA for functional studies were done using the site-directed mutagenesis using the QuikChange XL Site-Directed Mutagenesis kit (Agilent Technologies).

**Temozolomide and doxorubicin treatment.** Doxorubicin (Sigma-Aldrich) and temozolomide (Schering-Plough) were used at various concentrations (refer to figures) on cell lines with or without GATA4 for investigation of effects on cell cycle and apoptosis.

**Generation of stable cell lines.** The U87 TRE-GATA4-inducible system was established by transfecting U87 cells with rtTA (pTET-On advanced vector; Takara Bio Inc.) and GATA4 cDNA (clone SC124037; OriGene) was delivered using Fugene HD transfection reagent (Roche). pCMV6-XL4 with no cDNA insert was used as an empty vector control. Gene-specific siRNA (Integrated DNA Technologies and QIAGEN) and scramble siRNA controls (all-star scramble siRNA; QIAGEN) were delivered to cultured cells using HiPerFect transfection reagent (QIAGEN) to a final concentration of 20 nM. Please refer to Table S1 for all siRNA sequences. For transient knockdown of TP53, TP53 shRNA clones in pLKO.1 vectors were obtained from Thermo Fisher Scientific. Vectors were transfected into U87 cells using Fugene HD at a ratio of 3:1 Fu gene HD/DNA. TP53 full-length cDNA was a gift from M. Irwin (SickKids Hospital). P21(CIP) cDNA expression vector was purchased from SINDNET (SickKids Hospital).

**Bisulfitesequencing and methylation PCR.** DNA collected from cell lines or GBM operative samples was bisulfite treated and purified using the EZ DNA methylation kit (BisCarch; Zymogen). For MSP-PCR, 80 ng DNA was used for template using GATA4 MSP-specific primers. For Bisulfitesequencing, GATA4 Bsp-PCR-specific primers were used and DNA was gel extracted and purified. DNA was cloned into a PCRI TOPO 2.1 sequencing vector (Invitrogen). A minimum of 10 clones were analyzed per cell line or GBM samples with NHA and NHB used as controls. Data were analyzed using BeQ analyzer software (v2.0; Bock et al., 2005). PCR conditions and primers were as previously described (Guo et al., 2004).

**SNP marker analysis for LOH.** PCR primers spanning GATA4 intragenic SNP markers were used to amplify PCR products of ~200–300 bp. Tumours in which mutations were identified had several SNP markers analyzed and compared with SNP markers in patient matched blood. All sequences were verified in triplicate using three independent PCR reactions and sequencing reactions. See Table S1 for primer sequences.

**In vitro methylation assay and luciferase assays.** In brief, a 1-kb promoter fragment was amplified from a GATA4 BAC (GATA4 BAC: RP11-241B23; obtained from The Centre of Applied Genomics at SickKids Hospital). The GATA4 −1-kb promoter was cloned into a promoter less pGL4.0 luciferase construct (Promega) or was treated with Sss1 methyl transferase (New England Biolabs, Inc.) incubated at 37°C at 1 h, supplemented with 160 µM S-adenosylmethionine before being cloned into pGL4.0. The P21(CIP) Lux construct was obtained from B Vogelstein (John Hopkins University, Baltimore, MD) and the P15IN(Lux) construct from P. Chiao (M.D. Anderson Cancer Center). Primer sequences for site directed mutagenesis to the GATA sites in these constructs are available in Table S1.

Luciferase assays of various promoter luciferase constructs were performed as follows. 2 µg luciferase construct of interest and 100 ng of control Renilla (RLUC; Promega) was transfected into cell lines using Fugene HD. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Arbitrary luciferase units were normalized to Renilla luciferase values. Each transfection was repeated in triplicate.

**Real-time PCR analysis.** Total RNA isolation was performed using an RNA extraction kit total RNA (Qiagen; Qiagen). cDNA was synthesized from 100 ng of total RNA using the Quantitect RT kit which includes a DNase treatment (QIAGEN). Real-time qPCR was performed on 40 ng cDNA template in a final volume of 25 µl using the Chromo4 Real-Time PCR detector (Bio-Rad Laboratories) using SYBR green fluorescence. Real-time PCR data were analyzed using analysis software (Monitor 3.1.3; Option). Data analysis was done using the ΔΔCT method with HPRT1 as a reference/control gene.

**Soft agar assay for in vitro transformation.** Soft agar plates were poured with a base layer of 0.5% agar in 6-well culture plates. 105 cells of various modifications and control lines were counted and mixed with equal volume of 0.75% soft agar for the top layer. 4 ml DME + 10% FBS media was poured on top of the agar overlay and colonies were counted 14 d after. Soft agar experiments were repeated in triplicate.

**Cell death assay.** In brief, 105 T98G cells were plated onto 6-well dishes in 2 ml DME +10% FBS. Cells were treated with siRNA scramble control, two different APNG siRNAs, two MGMT siRNAs, or a combination of...
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APNG + MGMT siRNA siRNA delivery was accomplished by HiPerFect transfection reagent using the manufacturer’s guidelines. Cells were then treated with 100 µM temozolomide for 48 h. After incubation with temozolomide, cells were collected and analyzed for cell death assay using trypan blue. Cells were counted using the Vi-CELL (12-Sample Carousel) Cell Viability Analyzer (Beckman Coulter).

BTIC and neurosphere cultures. G179, G144, and nontransformed HF-240 cells were grown using serum-free media supplemented with 20 ng/ml G179, G144, and nontransformed BTIC and neurosphere cultures. 

Neurosphere assays were conducted by counting 1,000 cells each of NHA-Ras control, shRNA control, and GATA4 shRNA1, GATA4 shRNA2 and placing them into 6-well noncoated plates using the media mentioned in the previous section. Neurospheres were then passaged into fresh 6-well plates containing sterile microscope slides coated with laminin containing stem cell media supplemented with 1% FBS for differentiation. After a minimum of 4 d of attachment and spreading, immunofluorescence cytochemistry analysis using GFAP (1:300; Dako), 04 (1:200; Millipore), and TUJ1 (1:500; EMD) was performed to assess differentiation potential.

IHC. Paraffin-embedded blocks were cut into 5-µm sections and were dewaxed in xylene followed by rehydration in alcohol series. The tissues were subjected to antigen retrieval by pressure cooking for 20 min in citrate buffer, pH 6, followed by blocking of endogenous peroxidase in 0.3% H2O2. Specific dilutions of primary antibodies used are as follows: GATA4 monoclonal antibody (1:100; Santa Cruz Biotechnology, Inc.), TUJ1 monoclonal antibody (1:500; Millipore), NeuN antibody (1:200; Millipore), TP53 (1:100; Cell Signaling Technology), NESTIN (1:250; EMD), Ki-67 (1:150; Dako), GFAP (1:300; Dako), and Synaptophyse (1:200; Abcam).

Primary antibody incubation times were overnight at 4°C degrees and detection was performed using biotinylated secondary IgG antibodies for 30 min using the ABC reagent kit (Vector Laboratories) and DAB chromagen (Vector Laboratories). Sections were counterstained briefly in hematoxylin (Thermo Scientific). Hematoxylin and eosin (H&E) sections were stained using standard protocols (Eosin Yellow Solutions 1% wt/vol; Thermo Fisher Scientific).

Western blot analysis. Primary astrocytes/GBM cultures and cell lines were lysed with standard PLC lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). Protein concentration was determined using the BCA (bicinchoninic acid) assay (Thermo Fisher Scientific). 30 µg of protein lysates were loaded into 10 or 12% SDS-PAGE gels. Proteins were then transferred onto PVDF membrane (NEN Research Products) using a semi-dry transfer apparatus (Bio-Rad Laboratories). Membranes were probed for varying proteins at 1 h: GATA4 (44; 1:200; Santa Cruz Biotechnology, Inc.), β-actin (1:5,000; Sigma-Aldrich Inc.), P14KRAF (K-18; 1:200; Santa Cruz Biotechnology, Inc.), cyclin D1 (SC-H295; 1:400; Santa Cruz Biotechnology, Inc.), P16INK4A (1:250; Santa Cruz Biotechnology, Inc.), P14ARF (K-19; 1:200; Santa Cruz Biotechnology, Inc.), P21CIP1 (1:500; BD), MGMT (1:300; Vector Laboratories). Protein detection was performed using Chemiluminescence Reagent Plus (PerkinElmer).

Statistical analysis. All experiments were performed in triplicate with means and standard error of the mean or standard deviation subjected to Student’s t test for pairwise comparison or ANOVA for multivariate analysis. Significance was defined as P < 0.05. Analysis of patient survival was performed using Kaplan-Meier analysis in SPSS software (v15) using three separate tests: log-rank (Mantel Cox test) and Breslow (Generalized Wilcoxon and Tarone-Ware).

Additional experimental procedures. Cell Cycle, BrdU proliferation assays, caspase 3/7 assay, and ChiP assays were done using standard methods as previously described in detail (Guha et al., 1997; Ding et al., 2001; Shannon et al., 2005; Kannasaran et al., 2007; Agnihotri et al., 2009).

Online supplemental material. Fig. S1 shows loss of GATA4 transcript by RT-PCR and loss of GATA4 by promoter methylation. Fig. S2 shows that GATA4 mutations are associated with LOH of SNP’s flanking mutations found in GATA4. Fig. S3 demonstrates that loss of GATA4 promotes transformation in nontransformed astrocytes. Fig. S4 shows that reexpression of GATA4 induces cell cycle arrest and promotes ubiquitination of CCND1. Fig. S5 shows that GATA4 is associated with the GAF promoter and loss of GAF is associated with loss of GATA4. Overexpression of GATA4 reduces proliferation in the brain tumor–initiating cell line G179. Fig. S6 shows that GATA4 represses in vitro transformation in breast, prostate, and lung cancer cell lines. Fig. S7 shows that GATA4 promotes apoptosis with temozolomide. Table S1 reports all primers, shRNA, and siRNA sequences used in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20102099/DC1.

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REFERENCES
