Cancer-Specific Requirement for BUB1B/BUBR1 in Human Brain Tumor Isolates and Genetically Transformed Cells

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ABSTRACT

To identify new candidate therapeutic targets for glioblastoma multiforme, we combined functional genetics and glioblastoma network modeling to identify kinases required for the growth of patient-derived brain tumor-initiating cells (BTIC) but that are dispensable to proliferating human neural stem cells (NSC). This approach yielded BUB1B/BUBR1, a critical mitotic spindle checkpoint player, as the top-scoring glioblastoma lethal kinase. Knockdown of BUB1B inhibited expansion of BTIC isolates, both in vitro and in vivo, without affecting proliferation of NSCs or astrocytes. Mechanistic studies revealed that BUB1B's GLE2p-binding sequence (GLEBS) domain activity is required to suppress lethal kinetochore-microtubule (KT-MT) attachment defects in glioblastoma isolates and genetically transformed cells with altered sister KT dynamics, which likely favor KT-MT instability. These results indicate that glioblastoma tumors have an added requirement for BUB1B to suppress lethal consequences of altered KT function and further suggest that sister KT measurements may predict cancer-specific sensitivity to BUB1B inhibition and perhaps other mitotic targets that affect KT-MT stability.

SIGNIFICANCE: Currently, no effective therapies are available for glioblastoma, the most frequent and aggressive brain tumor. Our results suggest that targeting the GLEBS domain activity of BUB1B may provide a therapeutic window for glioblastoma, as the GLEBS domain is nonessential in untransformed cells. Moreover, the results further suggest that sister KT distances at metaphase may predict sensitivity to anticancer therapeutics targeting KT function. Cancer Discov; 3(2); 198–211. ©2012 AACR.

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INTRODUCTION

Glioblastoma multiforme is the most aggressive and common form of brain cancer in adults (1). Currently, no effective therapies are available for glioblastoma. Even with standard of care treatments, such as surgery, radiation, and chemotherapy, approximately 90% of adult patients die within 2 years of diagnosis (2). Both adult and pediatric brain tumors seem to be hierarchically organized, suggestive of a cancer stem cell origin (3–6). Consistent with this notion, brain tumor-initiating cells (BTIC) have recently been isolated that retain the development potential and specific genetic alterations found in the patient's tumor (3, 4, 7, 8). When implanted into the cortex of rodents, BTICs give rise to glioblastoma-like tumors with patient-specific molecular signatures and histologic features (5–8). Expression of neural progenitor molecular networks may contribute to the aggressive behavior of glioblastoma tumors through enhancing self-renewal or developmental programs (9), DNA repair pathways (10), angiogenesis (11), and/or invasiveness (12). Given the likelihood of BTIC-driven maintenance and spread of glioblastoma, effective cell-based therapies will likely have to target the stem cell.

Recently, a new method for deriving and maintaining BTICs was developed in which adult BTICs can be isolated and grown in serum-free, defined monolayer culture (7, 8). By this method, BTICs can retain tumor-initiating potential and tumor-specific genetic and epigenetic signatures over extended outgrowth periods (13). Here, we sought to take advantage of this BTIC culture system to find evidence for the cancer-lethal hypothesis: that transformed cells, compared with “normal” cells, harbor novel molecular vulnerabilities as a direct consequence of cancer-causing genetic alterations (14). Although multiple studies have addressed the question of cancer lethality in serum-derived cell lines (15, 16), there remain lingering questions of applicability to human cancers, as serum-derived lines may not faithfully represent the primary cancer (7).

By combining the results of short hairpin RNA (shRNA) kinome screens in BTICs and neural stem cells (NSC) for genes required for progenitor expansion with a glioblastoma bionetwork created from patient molecular signatures, we identified BUB1B, a critical mitotic checkpoint kinase (17), as the top glioblastoma-specific hit. Our results...
suggest that glioblastoma tumors and genetically transformed cells have an added requirement for BUB1B to suppress lethal consequences of altered kinetochore (KT) function. Importantly, these studies show that nontransformed cells do not require BUB1B/BubR1 for chromosome alignment, nor do they require the GLE2p-binding sequence (GLEBS) domain to maintain the spindle assembly checkpoint. They further suggest that altered KT conformations, apparent in glioblastoma and genetically transformed cells, may predict cancer-specific sensitivity to BUB1B inhibition and perhaps other mitotic targets that affect KT–microtubule (KT–MT) stability.

RESULTS

An RNA Interference Kinome Screen for Genes Differentially Required for BTIC Expansion

To discover candidate therapeutic targets for glioblastoma, we conducted an shRNA screen targeting 713 human kinases to identify gene activities required for in vitro expansion of BTICs. To enrich for BTIC-specific hits, a parallel screen was conducted in human fetal NSC-CB660 cells (Fig. 1A; ref. 18). NSCs share molecular and phenotypic features with BTICs, including identical isolation and growth in serum-free conditions, similar doubling times, overlapping expression profiles, and similar developmental potential (18). However, they retain a normal karyotype and are not tumorigenic (18) and, thereby, represent ideal controls for BTICs.

This screening approach (see Methods for details) revealed approximately 48 candidate kinase targets with shRNAs underrepresented in BTICs relative to NSCs (Supplementary Table S1). To prioritize these hits, we examined whether hits could be parsed into distinct pathways and/or complexes using protein–protein interaction networks (19). By this analysis, most hits were connected in a single, large subnetwork, enriched for 248 gene ontology (GO) biologic processes (multiple testing adjusted P < 0.01), such as protein kinase cascade (P = 5.57881e–085) and protein amino acid phosphorylation (P = 1.10068e–082). This lack of specific biologic processes likely reflected the fact that these kinases are well studied and involved in many biologic processes and, thus, did not provide any useful information for prioritizing of candidate hits.

As an alternative strategy, we examined the occurrence of screen hits in a glioblastoma-specific regulatory network, constructed de novo from more than 421 glioblastoma samples from The Cancer Genome Atlas (TCGA; ref. 20) by integrating gene expression and DNA copy number variation data (refs. 21, 22; Supplementary data). By this analysis, 37 of 48 shRNA candidate hits appeared as nodes in the glioblastoma network. Examination of subnetworks in the glioblastoma network revealed 15 biologic processes significantly enriched (5 cell-cycle related and 9 general phosphorylation related), including the M-phase of mitotic cell cycle (P = 1.64e–5). The largest glioblastoma-specific subnetwork contained 4 screen hits, including AURKB, BUB1B, MELK, and PLK1 (Fig. 1B).

To control for glioblastoma network comparisons, we also examined screen hits in a normal brain network constructed from 160 nondementia human prefrontal cortex samples. Only 20 of 48 candidate hits appeared in the normal brain network and produced smaller subnetworks enriched for general phosphorylation-related GO biologic processes (data not shown). Although BUB1B appeared in this network, it was connected to only one gene and had no down nodes (Fig. 1B), and thus was not a key driver node.

BUB1B Is Differentially Required for BTIC Expansion

Retests of AURKB, BUB1B, MELK, and PLK1 revealed that BUB1B inhibition gave the largest differential effect on BTICs from multiple glioblastoma isolates, including common developmental subtypes (24), without observable toxicity in proliferating NSCs or astrocytes (Fig. 1A–D). In these studies, shRNA-expressing cells were subjected to short- and long-term outgrowth assays (Fig. 2D and Supplementary Fig. S1A and S1B). Knockdown of KIF11 was used as a positive control. KIF11 is a microtubule motor protein required for mitotic progression in proliferating mammalian cells (13). During short- and long-term outgrowth, shKIF11 blocked the growth of BTICs, NSCs, and astrocytes. Because shKIF11 inhibits only cycling cells entering mitosis, shKIF11-dependent growth inhibition indicates similar division rates for various cells used and shows they have comparable RNA interference (RNAi) pathway activity. However, BUB1B knockdown also deleterious to BTIC tumor sphere formation, which may reflect tumor-initiating cell activity (ref. 25 and Supplementary Fig. S1C and S1D). BUB1B knockdown was also deleterious to BTIC tumor sphere formation, which may reflect tumor-initiating cell activity (5, 6) in both BTICs and primary tumor samples (Fig. 2E). However, knockdown did not profoundly alter the expression of SSEA1 or other progenitor markers, including Sox2 and Nestin, or neural lineage markers, including GFAP and TUJ1 (data not shown).

In contrast, PLK1 knockdown had a partial effect, MELK knockdown had no effect, and inhibition of AURKB was equally toxic to BTICs and NSCs (Supplementary Fig. S2A–S2C). On the basis of these results, we further pursued BUB1B as a candidate BTIC-lethal gene.

BUB1B Is Overexpressed in Glioblastoma Isolates, and Its Checkpoint Activity Is Compromised by shBUB1B in Both BTICs and NSCs

BUB1B is a highly conserved BUB1-like kinase, BubR1, whose activity is essential for mitotic spindle checkpoint signaling (17). The mitotic spindle checkpoint monitors the attachment of kinetochores to the plus ends of spindle microtubules and prevents anaphase onset until chromosomes are aligned and kinetochores are under tension at the metaphase plate (17). Because of its role in maintaining chromosome stability, mitotic spindle checkpoint activity has been touted as a mechanism for tumor suppression (17, 26). In rare instances, partial loss-of-function mutations in checkpoint genes have been reported for certain cancers (26). However, many late-stage cancers, including glioma, exhibit high BUB1B expression (27, 28), suggestive of hyperactivity.

To begin to reconcile these observations with our results, we analyzed BUB1B expression patterns and activity in
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BTICs and NSCs. We observed that BUB1B and other spindle checkpoint genes were upregulated in BTIC isolates and also RAS-transformed astrocytes, as judged by mRNA and protein abundance (Supplementary Fig. S3A–S3C). Moreover, both BTICs and NSCs had normal mitotic spindle arrest responses after paclitaxel treatment, which were abrogated by BUB1B knockdown (Supplementary Fig. S4A–S4C). Thus, BUB1B knockdown achieves a similar level of suppression of BUB1B mRNA, protein, and activity in both BTICs and NSCs. The results suggest that BUB1B knockdown produces a hypo-morphic state to which BTICs, but not NSCs or astrocytes, are sensitive. Later, we provide further evidence to support this conclusion, by addressing BUB1B’s essential and nonessential functions in BTICs and in transformed and untransformed cells.

**Shortened Interkinetochore Distances Are Indicative of Sensitivity to shBUB1B in BTICs and Genetically Transformed Cells**

One possible explanation for BTIC’s observed sensitivity to hypomorphic BUB1B activity is that KT–MT dynamics could be...
Figure 2. BUB1B is validated as a candidate glioblastoma-lethal gene in vitro. A, BTIC-specific effects of BUB1B knockdown, visualized using shRNA–green fluorescent protein (GFP) BTICs and NSCs 6 days after posttransduction with pGIPz-shRNA virus. Knockdown of KIF11/EG5, a microtubule motor protein critical for bipolar spindle formation during mitosis, was used as a positive control for both RNAi pathway activity and cell proliferation.

B and C, examination of BUB1B knockdown by Western blot and quantitative real-time PCR (qRT-PCR) analysis in BTIC-G166 and NSC-CB660 cells.

D, comparison of the effects of BUB1B knockdown on in vitro expansion of multiple BTIC and NSC lines and normal human astrocytes (NHA). **, Student t test; P < 0.01. See Methods for a description of how BTIC isolates were developmentally subtyped.

E, limiting dilution assays (LDA) for in vitro tumor sphere formation. BTIC-0131 cells and also unpassaged primary glioblastoma tumor cells (448T) were transduced with indicated LV-GFP-shRNAs, diluted, and assayed for sphere formation after 14 days. Linear regression analysis was used to evaluate significance.
altered to favor MT detachment. To properly segregate chromosomes during mitosis, stable attachments must occur between the “plus” end of mitotic spindle MTs and KTs, which are formed at the centromeres of each sister chromatid as cells enter mitosis (29). Early in mitosis, KT–MT attachments are unstable and dynamic, allowing chromosomes to be towed toward MT plus ends during congression and improperly attached chromosomes to be released and reattached to spindle MTs until they are bioriented and under tension (19, 29). The distance between KTs on sister chromatids can range from approximately 0.6 μm in prophase to more than 1 μm in metaphase, when sister KTs achieve stable MT attachment and are pulled toward opposing spindle poles (Fig. 3A; ref. 30). In the prevailing model, this KT movement prevents outer kinetochore proteins from being phosphorylated by Aurora B kinase, which promotes KT–MT detachment (e.g., for error correction) by physically removing them from centromere-embedded Aurora B activity (29).

To examine KT–MT dynamics, we first investigated the possibility that KT dynamics may be altered in glioblastoma cells. To this end, we measured interkinetochore distance (IKD), the maximum distance achieved between sister KTs when stable MT attachment has occurred (31). We first measured IKDs for shBUB1B-insensitive NSCs (CB660) and 2 shBUB1B-sensitive BTIC isolates (G166 and 0131). The results were surprising. We found that IKDs were significantly shorter in both BTIC isolates (1.23 μm for CB660 vs. 1.13 μm for G166 and 1.09 μm for 0131; Fig. 3B and C). Thus, BTIC IKDs were shorter by 100 to 140 nm or 50 to 70 nm for each sister KT. This finding represents a significant change, as, for example, the outer kinetochore protein Hecl moves approximately 40 nm toward the spindle pole as KTs come under tension (32, 33).

Next, we examined IKDs in 2 glioblastoma patient isolates, 0827 and 1502, which we had observed were completely insensitive to shBUB1B. These isolates were insensitive despite having similar knockdown efficiencies to shBUB1B-sensitive lines and among the fastest doubling times and tumor initiation rates (data not shown). Measuring IKDs in these cells revealed that they were indistinguishable from NSCs (1.23 μm), suggesting the possibility that IKDs may predict BUB1B sensitivity (Fig. 3B and C).

To further examine this possibility, we tested a hypothesis that shortened IKDs and added BUB1B requirement arise as a result of oncogenic transformation and, specifically, oncogenic signaling. It was recently shown that expression of activated Ras oncogene can lead to mitotic stress and induce chromosome instability in mammalian cells, through an as yet undefined mechanism (16). Thereby, we examined IKDs in p53−/− mouse embryo fibroblasts (MEF) with or without RasV12 expression. In p53−/− control MEFs, IKDs averaged 1.25 μm, similar to those of NSCs and 827 cells. Surprisingly, RasV12 expression converted long IKDs to short, averaging 1.13 μm, indistinguishable from those of G166 and 0131 cells (Fig. 3D). Moreover, RasV12 transformation also converted MEFs from being resistant to BUB1B inhibition to being profoundly sensitive, which was true for human astrocytes (Fig. 3D) as well (both experiments are presented below in Fig. 5 and Supplementary Fig. S8A–S8C). Importantly, all of the IKD measurements for BTICs, NSCs, and MEFs were scored blindly to avoid experimenter bias.

Because most BUB1B/BubR1 experimentation has been carried out in HeLa cells, which are derived from a cervical carcinoma (16), we next measured IKDs in these cells. As a control, we used immortalized retinal pigment epithelial (RPE-1) cells, which are untransformed. HeLa cells showed IKDs similar to other BUB1B-sensitive cells (1.11 μm), whereas RPE cells showed long IKDs, similar to those of insensitive cells (1.22 μm). In repeating the pattern above, BUB1B knockdown affected chromosome dynamics only in HeLa cells (detailed later).

These results suggest (i) that IKDs occur in discrete intervals: long (∼1.24 μm) and short (∼1.12 μm); (ii) that short IKDs predict sensitivity to BUB1B inhibition; and (iii) that RasV12 transformation is sufficient to induce short IKDs and sensitivity to BUB1B.

Glioblastoma Isolates with Short IKDs Require BUB1B to Suppress Severe KT–MT Attachment Defects

We next wished to determine whether BTICs with short IKDs have altered KT–MT dynamics that favor detachment. To this end, we used a metaphase chromosome alignment assay, in which KT–MT attachment defects are visualized as misaligned chromosomes during metaphase arrest induced by proteasome inhibition (34). By this assay, knockdown of BUB1B resulted in dramatic chromosome alignment defects only in BTICs with short IKDs but did not affect alignment in NSCs, 0827 cells (Fig. 4A and B; Supplementary Fig. S5A), or astrocytes (see later). The alignment defects in G166 cells were accompanied by profound loss of KT–MT attachment, as indicated by lack of colocalization of KTs with cold-resistant MTs (Supplementary Fig. S5B). Moreover, examination of phospho-Ser44-Hec1/Ndc80 at KTs revealed that after BUB1B knockdown, G166s retain Ser44 phosphorylation (ref. 35; Supplementary Fig. S5C). This phosphorylation is dependent upon centromere-embedded Aurora B kinase activity and has a KT–MT destabilizing effect (29). These results suggest that cells with short IKDs have KT–MT attachment defects, which BUB1B is required to suppress.

Consistent with this idea, G166 cells also displayed overt differences in chromosome dynamics during mitosis, with significantly more lagging chromosomes in anaphase than with NSCs (Fig. 4C). BUB1B knockdown dramatically exacerbated these defects (Fig. 4D). In control experiments in NSCs, shBUB1B did not affect lagging chromosome frequency or karyotype after extended outgrowth (Fig. 4C and Supplementary Fig. S5D), again suggesting that cells with long IKDs do not use BUB1B in the same way.

Genetic Dissection of the Added Requirement of BUB1B in RasV12-Expressing Fibroblasts and BTICs

BUB1B has multiple functional domains that have been implicated in mitotic checkpoint control, mitotic timing, and stable KT–MT attachment (17, 29). These include N- and C-terminal KEN box domains required for Cdc20 binding and anaphase-promoting complex (APC) inhibition (36–38); a C-terminal kinase domain involved in checkpoint control (39, 40); and a GLEBS-like motif necessary for KT localization during mitosis (refs. 39, 41; Fig. 5A). Although BUB1B is necessary for mammalian development (42), its essential function is contained solely within the N-terminal KEN box (36), which enables BUB1B/BubR1 to act as a pseudo-substrate inhibitor of APC/C-Cdc20 during G2 and preanaphase mitosis, preventing a precocious anaphase (36).
**Figure 3.** Measurement of IKD in BUB1B-resistant and -sensitive cells. **A,** diagram showing IKD measurement. **B,** measurement of IKDs in BTICs, NSCs, MEFs, MEF-Ras cells, RPE cells, and HeLa cells, using immunofluorescent staining of kinetochores. Constitutive centromere-associated network (CCAN/CREST) proteins (red) and outer kinetochore protein, Hec1 (green) were visualized to identify kinetochore pairs. IKDs were measured between Hec1 centroids, using Applied Precision softWoRx software package. **C** and **D,** quantification of IKDs from **B.** *, P < 0.001 by Student t test.
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The above results suggested that BTICs with short IKDs have an added requirement for BUB1B that helps facilitate KT–MT attachment. The GLEBS domain of BUB1B is necessary for its KT localization and interaction with BUB3, and helps facilitate KT–MT attachment (39, 41). Maulureanu and colleagues (36) have shown that this domain is nonessential for stable end-on KT–MT attachment and viability in MEFs. Their results, however, were not consistent with previous work in HeLa cells that clearly showed that the GLEBS domain is essential for KT–MT attachment (43). Intriguingly, our datasets inform these seemingly incompatible results with the following thesis: The GLEBS domain of BUB1B is required in cells with abnormal KT conformations (e.g., HeLa cells) to suppress lethal KT–MT instability. Furthermore, as our results earlier show that RasV12 transformation can convert long IKDs to short ones, it would follow that oncogenic transformation gives rise to added BUB1B requirement.

To directly address this idea, we next conducted allelic complementation studies using mouse Bab1b alleles (Fig. 5A) in MEFs harboring biallelic deletion of Bab1b (36), which were also transformed by H-RasV12. For these experiments, we used full-length (FL) mouse Bab1b, the N-terminal deletion mutant, and the E406K GLEB domain–mutant allele, which cannot bind to KTs. Expression of each allele was verified by Western blotting (Supplementary Fig. S6A–S6C). For nontransformed MEFs, the viability pattern was the same as previously published (36): Both FL and E406K alleles fully complemented Bab1b+/− and only the N-terminal KEN domain–mutant allele, which cannot bind to KTs. Expression of each allele was verified by Western blotting (Supplementary Fig. S6A–S6C). For nontransformed MEFs, the viability pattern was the same as previously published (36): Both FL and E406K alleles fully complemented Bab1b+/− and only the N-terminal KEN box domain was required for Bab1b+/− cell growth (Fig. 5B). However, after RAS-dependent transformation of these cells, the results changed dramatically. The GLEBS domain became essential for viability, as evidenced by the complete failure of the E406K allele to complement (Fig. 5B). RasV12 activity, however, did not alter the requirement for the N-terminal KEN box domain. These results show that RasV12 transformation...
 leads to a profound requirement for BUB1B's GLEBS domain activity.

To further examine this result, we conducted a similar set of complementation experiments in BTIC-G166 cells, using mouse Bub1b alleles to complement knockdown of endogenous human BUB1B. In this case, kinase-defective and internal KEN box deletion alleles were also included (Fig. 5C). Expression of each allele was confirmed by Western blotting (Supplementary Fig. S6A and S6B). Knockdown efficiency of endogenous BUB1B was also shown to be unaffected by the expression of mouse Bub1b (shBUB1B is not predicted to target mouse Bub1b; Supplementary Fig. S6C). As shown in Fig. 5C, expression of full-length and KD2 mBub1b alleles achieved near-complete rescue of the viability defects of BUB1B knockdown in G166 cells, indicating that the shBUB1B phenotype is due to on-target silencing of BUB1B, and that the kinase activity of BUB1B is not required in BTICs. In contrast, the ∆N and E406K alleles failed to complement viability, behaving exactly as the vector control, whereas ∆M showed a partial rescue (Fig. 5C). None of the alleles were able to complement control KIF11 knockdown. Thus, these results phenocopy those observed in RasV12-transformed MEFs.

To further investigate these results, we conducted KT–MT attachment using chromosome alignment assays as described above. All alleles showed complete or partial suppression of alignment defects, except for E406K, which failed to complement (Fig. 5D and E). This observation indicates that the

**Figure 5.** Allelic complementation studies with mouse Bub1b (mBub1b) mutants in Bub1b−/− MEFs and BTICs. A, the mouse alleles used in these studies were previously published and include FL, KD2, which harbors 2 point mutations in the kinase domain (K784R in the ATP binding domain and K802R in the catalytic domain). ∆N, which lacks the N-terminal Cdc20-binding domain 1; ∆M, which lacks the C-terminal Cdc20-binding domain 2; and E406K, which creates a point mutation in the GLEBS motif that interferes with kinetochore localization and Bub3 binding. B, viability assessment of complementation studies using p53−/− MEFs with floxed alleles of Bub1b, with and without transformation via H-RasV12. Knockdown of BUB1B in RasV12-transformed NHAs phenocopies the BUB1B requirement observed in BTICs with respect to viability. MEFs were transduced with murine stem cell virus (MSCV)-GFP-mBub1b constructs, sorted for GFP+ outgrowth, and seeded into microtiter growth dishes for proliferation assays.

C, viability assessment of complementation studies using BTIC-G166 with shBUB1B (or controls) expressing each of 5 mBub1b alleles from A. Assays were conducted as in Fig. 1C, D, and E, chromosome alignment after complementation of BUB1B knockdown with mBub1b alleles, as in Fig. 4A and B. Scale bar, 10 μm.
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Figure 6. BUB1B knockdown inhibits BTIC-dependent tumor growth. A, orthotopic xenotransplants of 131 BTIC cells after stable transduction with shControl or shBUB1B. Top and bottom, experimental NSG mice 4 and 7 weeks after injection, respectively. Right, light images of brains from control. Middle, GFP+ fluorescence marking shRNA-containing cells. Left, fluorescent signal from chlorotoxin (CTX) Cy5.5 conjugate, marking bulk tumor mass. Results indicate that GFP-expressing shBUB1B cells were unable to contribute to the formation of orthotopic tumors and yielded tumor masses dominated by wild-type control cells with little to no detectable GFP expression. Quantification of GFP fluorescence in tumor 0131 orthotopic xenotransplants is shown in Supplementary Fig. S9. B, survival plots for mice with BTIC-0131 brain xenografts, with or without knockdown of BUB1B. Median survival for shCtrl = 178 days; shCtrl, n = 7; shBUB1B, n = 6.

**ShBUB1B Inhibits BTIC-Driven Tumor Formation**

Finally, to ensure that the above results are applicable to tumor formation in patients, we examined the BUB1B requirement during BTIC tumor formation, for a BTIC line with short IKDs. We conducted 2 different assays. In the first, shRNA-GFP 0131 cells competed against non-shRNA control 0131 cells at an approximately 9:1 ratio (Fig. 6A) for injection into the cortex of immunodeficient mice. The endpoint was relative representation of shBUB1B. After 4 or 7 weeks post injection, control cells had dramatically outcompeted shBUB1B-GFP+ cells (Fig. 6A). This finding was not simply due to the inviability of injected cells, as most of the shBUB1B-GFP+ cells in the injection bolus could attach to laminin-coated dishes. For the second assay, survival was the endpoint for mice injected with either shControl or shBUB1B-expressing 0131 cells (Fig. 6B). This assay ended at 250 days after injection, when 90% of control mice had succumbed to tumors. During this time, none of the shBUB1B-0131 mice died. These results suggest that knockdown of BUB1B is deleterious to glioblastoma tumor formation and that the in vivo tumor environment does not suppress requirement for BUB1B.

**DISCUSSION**

Here, we attempted to identify kinases differentially required for the expansion of glioblastoma-derived BTICs by combining a functional genetic approach with a glioblastoma bionetwork derived from sample molecular datasets from patients (44). This approach produced BUB1B as the top-scoring screen hit. Validation studies bore out this prediction: Knockdown of BUB1B differentially blocked expansion of 9 of 11 BTIC isolates examined, without affecting...
growth of human NSCs and astrocytes, which are both candidate cell types of origin for glioblastoma (44).

BUB1B is a highly conserved BUB1-like kinase, BubR1, the activity of which is essential for mitotic spindle checkpoint signaling (17). The mitotic spindle checkpoint monitors the attachment of KTs to the plus ends of spindle MTs and prevents anaphase onset until chromosomes are aligned and kinetochores are under tension at the metaphase plate (17). To pursue the mechanism of BUB1B requirement in glioblastoma cells, we tested a hypothesis: that KT–MT dynamics are fundamentally altered in glioblastoma cells to favor KT–MT detachment, which BUB1B is required to suppress.

This hypothesis was supported by multiple observations (summarized in Fig. 7). First, in examining sister KT dynamics at metaphase, we showed that glioblastoma and other cancer cells sensitive to BUB1B inhibition have significantly shorter IKDs, indicating that KT dynamics are dramatically altered. Second, in BTICs with short IKDs, BUB1B activity is required to suppress lethal KT–MT instability and to directly or indirectly inhibit centromere-embedded Aurora B activity on outer KT proteins. Third, expression of the RasV12 oncogene is sufficient to induce the same changes in sister KTs observed in BTICs. Fourth, RasV12 also triggered requirement for the GLEBS domain of BUB1B for both viability and KT–MT attachment. Fifth, genetic dissection of BUB1B function in BTICs with short IKDs revealed the same requirement for the GLEBS domain of BUB1B to suppress lethal KT–MT instability.

These results support a model whereby oncogenic signaling alters KT regulation, resulting in short IKDs and KT–MT instability. As a direct result, BUB1B’s GLEBS domain activity becomes essential for KT–MT attachment. In the absence of GLEBS domain activity, cells with short IKDs undergo mitotic catastrophe and are inviable (Fig. 7). Importantly, our studies show that nontransformed cells do not require BUB1B for chromosome alignment, nor do they require the GLEBS domain to maintain the spindle assembly checkpoint or viability.

One key implication of this work is that short IKDs may be predictive of the requirement for the GLEBS domain of BUB1B and sensitivity to disruption of KT function in cancer cells. For example, we have found glioblastoma isolates (i.e., 0827 and 1502) from patients that are resistant to BUB1B knockdown and have IKDs indistinguishable from those of untransformed cells. Thus, it is conceivable that anticancer therapies targeting KT or mitotic checkpoint function (e.g., refs. 45 and 46) would benefit from using IKDs as a biomarker or companion diagnostic. However, additional studies are required to determine the extent to which IKDs are shorter in cancer cells and also the mechanisms through which KT conformations become perturbed.

One possibility is that RTK-Ras signaling directly affects KT function. Evidence has come to light that Ras effector kinases Erk1/2 can directly phosphorylate the C-terminal domain of CENPE, a key KT protein, which is predicted to decrease its MT-binding ability (47, 48). Intriguingly, we observed that in both BTIC-G166 and RasV12-transformed astrocytes, which have short IKDs, significant upregulation of Erk1/2 activity takes place in prophase and mitosis (Supplementary Fig. S9). Thus, it is conceivable that inappropriate regulation of the RTK-Ras pathway in mitosis could directly affect KT–MT attachments and/or KT conformational states. Although RasV12-transformed astrocytes may not faithfully recapitulate the mutation spectra of glioblastoma (only ∼2% have mutant Ras activity; ref. 20), the Ras pathway is predicted to be inappropriately activated in a majority of glioblastoma tumors (ref. 20; e.g., by NF1 mutation or RTK activity). Future work is required to examine the relationship between Ras signaling and KT regulation.

Our results also shed light on a recent study that identified genes differentially required in cancer cell lines overexpressing the activated KRAS oncogene (16). Their results suggest that activated RAS oncogene activity triggers differential requirement for a PLK1-associated mitotic network (16), which they
proposed resulted from Ras-induced “mitotic stress.” Our results suggest that these phenotypes likely result from KT–MT attachment defects arising from KT conformational abnormalities.

Finally, our studies also inform the use of a large collection of cancer patient molecular signatures. We used more than 300 glioblastoma patient molecular signatures to create a Bayesian bioworket, which, when combined with our functional genetic data, predicted BUB1B inhibition to be differentially lethal for glioblastoma cells. To our knowledge, this is the first time a bioworket derived solely from patient data has been used to successfully predict gene activity specifically required for cancer cells. Intriguingly, integrating our BTIC kinome dataset into a bioworket for breast cancer also yielded BUB1B as the top-scoring hit (J. Zhu; personal communication), suggesting that our results should prove useful for other cancers.

In summary, our results suggest that glioblastoma tumors and genetically transformed cells have an added requirement for BUB1B to suppress lethal consequences of altered KT function. They further suggest that IKDs may predict cancer-specific sensitivity to BUB1B inhibition and perhaps other mitotic targets that affect KT–MT stability.

**METHODS**

**shRNA Barcode Screens and Array Analysis**

For shRNA screen and barcode array analysis, cells were infected with a pool of lentiviral shRNAs targeting 713 human kinases at a representation of approximately 1,000-fold [multiplicity of infection (MOI) < 1]. At day 3 post infection, an initial day 0 sample was taken. The rest of the population was selected with puromycin (Sigma; 2 μg/mL) to remove uninfected cells. Afterwards, cells were propagated in culture for an additional 21 days and sampled for barcode array analysis at 21 days. For each passage, a minimal representation of 1,000-fold was maintained. For each corresponding sample, shRNA barcodes were PCR recovered from genomic samples, labeled with Cy5 or Cy3, and competitively hybridized to a microarray containing the corresponding probes (Agilent Technologies). Replicate array results were analyzed using the BioConductor package limma. The change in the relative abundance of each shRNA in the library over time was measured using the normalized Cy3/Cy5 ratio of its probe signal. Barcode probes depleted in the BTIC samples were considered candidate genes, using the following criteria: (i) adjusted P ≤ 0.05 and (ii) log2(ratio) ≥ 0.585.

**Cell Culture**

BTIC and NSC lines used in these studies have been previously published (7, 8) and were grown in N2B27 neural basal media (STEMCELL Technologies) supplemented with EGF and fibroblast growth factor 2 (FGF-2) of 20 ng/mL each (Peprotech), on laminin-coated polystyrene plates (Sigma) and passaged according to Pollard and colleagues (8). Immortalized CX cells and VM cells (Millipore) were maintained in ReNcell maintenance medium with EGF and FGF-2 (20 ng/mL each; Peprotech) and also grown on laminin-coated tissue culture–treated plates and passaged according to Pollard and colleagues (8). NHA (STEMCELL Technologies) and NHA-Ras cells (Russell Peper, University of California San Francisco, San Francisco, CA) were grown in astrocyte growth medium (Clonetics) according to the manufacturer’s instructions and published protocols (49).

**RNAi**

The shRNAs were obtained from the RNAi Shared Resource [Fred Hutchinson Cancer Research Center (FHCRC)] or Open Biosystems in the pGIPZ lentiviral vector. Target sequences for shRNAs are as follows: BUB1B, #1, CDS:1417, CCTACAAAGGAGAACACTA; BUB1B, #2, CDS:1547, AGGAAACACCTCCTTTCCA; and KIF11, CDS:571, AAGAGGAGGATATATA. For virus production, pGIPZ-shRNA plasmids were transfected into 293T cells along with psPAX and pMD2.G packaging plasmid to produce lentivirus. Approximately 24 hours after transfection, NSC expansion medium was added to replace original growth medium. Virus was harvested 24 hours after medium change and stored at −80 °C. BTICs and NSCs were infected at MOI < 1 and selected with 2 to 4 μg of puromycin for 2 to 4 days.

**qRT-PCR**

QuantiTect quantitative real-time PCR (qRT-PCR) primer sets and QuantiFast SYBR Green PCR Kits (standard) were used according to the manufacturer’s instructions with the ABI PRISM 7900 Sequence Detection System (Genomics Resource, FHCRC). Relative transcript abundance was analyzed using the 2−ΔΔC T method. TRIzol (Invitrogen) extraction was used to collect total RNA from cells.

**Western Blot Analysis**

Western blots were carried out using the standard laboratory practices, except that a modified radioimmunoprecipitation assay buffer was used for protein extraction (5% sodium deoxycholate, 1% SDS, 4% DOC, 4% Triton-X 100, 2 mM LiDTP) and complete protease inhibitors (Roche) followed by a 15-minute digestion with 125 units of Benzonase (Merck) at room temperature. The following antibodies were used for detection: BUB1B (1:1,000; Sigma), Actin (1:1,000; Cell Signaling), and cleaved PARP (1:1,000; Cell Signaling). An Odyssey infrared imaging system was used to visualize blots (LI-COR) following the manufacturer’s instruction.

**Growth Assays**

For short-term outgrowth assays, after selection, shRNA-transduced cells were harvested, counted (NucleoCounter, NBS), and plated onto a 96-well plate. After 7 days under standard growth conditions, the cell proliferative rate was measured using AlamarBlue reagent (Invitrogen). For long-term outgrowth assays, after selection, shRNA-transduced cells were mixed with nontransduced cells (9:1) and outgrown for 14 to 24 days using our standard passaging protocol. The GFP fraction, which marks shRNA-containing cells, of each population was measured by fluorescence-activated cell sorting (FACS; BD LSRS flow cytometer; FHCRC Shared Resources) at 5- to 8-day intervals.

**Spindle Checkpoint Arrest**

For image-based assessment, cells were plated in a 96-well plate and then treated with paclitaxel (Sigma) and nocodazole (Sigma) for various time points (6–18 hours). After treatment, cells were fixed with 2% paraformaldehyde for 30 minutes, permeabilized with 0.25% Triton X-100, and blocked in PBS containing 3% bovine serum albumin and 5% goat serum. After 3 washes with PBS, cells were stained with MPM-2 (1:300, Millipore) at room temperature for 1 hour. Next, cells were washed and incubated with Alexa Fluor 568 secondary antibody (Invitrogen) and DAPI for 1 hour in the dark. Staining was visualized by the Nikon Eclipse Ti microscope. For FACS-based assessment, cells were then collected and fixed in 70% ethanol for 1 hour at 4°C, then rinsed with ice-cold PBS + 2% fetal calf serum, and stained with anti-MPM-2 (1:300, Millipore), anti-mouse Alexa Fluor 568 (1:200, Invitrogen), and DAPI (1 μg/mL). Cells were washed, resuspended in PBS, and filtered. The mitotic index was measured by a BD LSRS flow cytometer (FHCRC Shared Resources).

**Mitotic Transit Time**

NSC- and BTIC-derived cells were transduced with control and BUB1B shRNA, respectively. After selection, cells were plated into a 96-well plate for time-lapse microscopy. During imaging, the atmosphere was...
maintained at a temperature of 37°C and 5% CO₂. Imaging was conducted using a Nikon Eclipse Ti microscope equipped with a live imaging system. NIKON Elements software was used to collect and process data. Images were captured at 5.3-minute intervals for 16 hours.

**Lagging Chromosome Assay**

For one-step arrest in prometaphase, cells were treated overnight with the EG5 kinesin inhibitor monastrol (100 μM/mL final concentration) overnight. DAPI staining was conducted to visualize the abnormal anaphase frequency. Monastrol inhibits the mitotic kinesin EG5KIF11, a motor protein required for spindle bipolarity, and specifically arrest cells in G2-M (26, 34). Cells were washed and released into fresh media for 2 hours and then fixed (4% PF), permeabilized, stained with DAPI, and visualized using a Nikon Eclipse E800 (Scientific Imaging, FHRC). More than 400 nuclei were counted for each trial (n = 5) and the Student t test determined significance. For asynchronous populations, cells were additionally stained with an MPM-2 antibody (Millipore), which marks mitotic cells, and counterstained with DAPI (Sigma). Approximately, one-third of MPM-2–positive cells in asynchronous cultures were in anaphase/telophase, whereas the other two thirds were in prometaphase or metaphase.

**Chromosome Alignment Assays**

For metaphase staining, cells were treated by 10 μM/L MG-132 (Tocris Bioscience) for 2 hours to arrest them at metaphase and then fixed for 20 minutes at room temperature with 4% formaldehyde in PBS and 0.2% Triton X-100. For cold-stable microtubules, cells were incubated on ice for 15 minutes before fixation. After fixation, cells were blocked and stained with α-tubulin (DM1A; 1:1,000; Sigma) and CREST anti-serum (1:1,000; Immunovision) at room temperature for 1 hour. Cells were washed and incubated with secondary antibody and DAPI for 1 hour in the dark. Immunolabeled cells were imaged on a DeltaVision RT deconvolution microscope (Applied Precision Inc.). Optical sections were acquired at 0.2-μm spacing with an Olympus ×100/1.4 NA UPLS Apo objective. Three-dimensional (3D) image stacks were deconvolved with Applied Precision’s proprietary software softWoRx, using a constrained iterative algorithm. Deconvolved 3D data were loaded into the visualization software Velocity (PerkinElmer). The number of misaligned MT-attached KTs was counted on the basis of CREST staining on 3D rendered images, and confirmed by visual inspection of maximum intensity projections of whole cells. Misaligned kinetochores were defined as those with normalized distance less than 0.2 μm. At least 30 cells were analyzed for each RNAi experiment.

**Xenotransplantation**

BTIC isolates (0131 cells) were infected with pGIPZ-shRNA virus and selected for 3 days in puromycin (2 μg/mL), so that more than 80% of cells were GFP+. Cells were then harvested using Accutase (Sigma), counted, resuspended in an appropriate volume of culture medium, and kept on ice before immediate transplantation. Nodonless diabetic/severe combined immunodeficient (NOD/SCID) IL2R- null mice (Jackson Laboratories #005557) were anesthetized by intraperitoneal injection of 0.2 mL/10 gm 1.25% Avertin solution and kept at 37°C. A small-bore hole was made in the skull, using a hand drill with a Meisinger #009 steel burr bit (Hager & Meisinger GmbH). A total of 2 × 10⁶ cells were slowly injected by pipette into the right frontal cortex approximately 2 mm rostral to the bregma, 2 mm lateral, and 3 mm deep through a 0.2- to 10-μL disposable sterile aerosol barrier tip (Fisher Scientific #02-707-30). The burr hole was closed using SURGIFOAM (Johnson & Johnson) and the skin rejoined using TISSEEL (Fisher Scientific #02-707-30). The burr hole was closed using SURGIFOAM (Johnson & Johnson) and the skin rejoined using TISSEEL (Fisher Scientific #02-707-30). The number of misaligned MT-attached KTs was counted on the basis of CREST staining on 3D rendered images, and confirmed by visual inspection of maximum intensity projections of whole cells. Misaligned kinetochores were defined as those with normalized distance less than 0.2 μm. At least 30 cells were analyzed for each RNAi experiment.

**Brain Tumor Imaging**

Seven weeks after the initial transplantation, mice were injected intravenously with 50 μL of 40 μM/L chlorotoxin: Cy5.5 conjugate (50) 2 hours before sacrifice by CO₂ inhalation. The brain and tumor were removed from the skull and imaged for Cy5.5 and GFP fluorescence using the Xenogen IVIS Spectrum imaging system (Caliper Life Sciences). Additional methods can be found in the Supplementary data.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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