REVIEW

Emerging insights into the molecular and cellular basis of glioblastoma

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Glioblastoma is both the most common and lethal primary malignant brain tumor. Extensive multiplatform genomic characterization has provided a higher-resolution picture of the molecular alterations underlying this disease. These studies provide the emerging view that "glioblastoma" represents several histologically similar yet molecularly heterogeneous diseases, which influences taxonomic classification systems, prognosis, and therapeutic decisions.

In his effort in the 1920s to cure two comatose patients with extensive glioblastomas, neurosurgeon Walter Dandy took the radical step of removing the entire affected hemisphere in each of these patients. Despite these heroic interventions, both of these patients succumbed to their disease due to involvement of the contralateral hemisphere (Dandy 1928). More than 80 years later, we continue to face the same clinical challenges illustrated by this vignette from the early 20th Century. Glioblastoma represents the most common primary intrinsic malignant brain tumor diagnosed each year in the United States; there are ~10,000 new diagnoses annually, and >50,000 patients are currently living with the disease (Davis et al. 2001; Porter et al. 2010; CBTRUS 2011). The clinical hallmarks of glioblastoma are its aggressive growth and inexorable recurrence despite multimodal therapy with surgery followed by radiation and temozolomide therapy. Unfortunately, current standard-of-care therapy results in a median survival of only 12–15 mo (Stupp et al. 2005).

The emergence of a molecularly focused approach to cancers represents a profound shift in our approach to the diagnosis and treatment of malignancy. This framework centers on a new taxonomy that describes cancers by their molecular alterations and the identification of inhibitors that target these cancer-specific changes. Indeed, therapies targeting *HER2*-amplified breast cancer (Slamon et al. 2001), CML harboring the *BCR–ABL* translocation (Druker et al. 2001), mutant *EGFR* lung cancer (Lynch et al. 2004), lung cancer harboring the *EML4–ALK* translocation (Kwak et al. 2010), and *BRAF* mutant melanoma (Chapman et al. 2011) provide clear proof of principle for this approach. Histopathologic diagnosis is increasingly being supplemented with annotation of amplification or mutational status where relevant (MacConaill et al. 2009; Dias-Santagata et al. 2010), and these data subsequently inform therapeutic decision-making, all in a time frame from target discovery to therapy that is becoming progressively shorter (Chabner 2011). However, despite clinical progress across many cancer types and extensive characterization of genomic alterations in glioblastoma, we still have not identified and exploited clinically meaningful tumor dependencies in this dreaded disease.

The recent characterization of the genome (Beroukhim et al. 2007; The Cancer Genome Atlas Research Network 2008; Parsons et al. 2008) and transcriptome (Phillips et al. 2006; Verhaak et al. 2010) of glioblastoma provides a high-

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resolution picture of the glioblastoma landscape that has revealed the major structural and expression alterations that may drive disease pathogenesis and biology. These comprehensive data sets reveal "glioblastoma" as a heterogeneous collection of distinct diseases with multiple dependencies both within and across each particular subtype. Here we summarize recent efforts to catalog the structural genomic landscape of glioblastoma and focus on emerging insights into critical molecular pathways central to glioblastoma pathobiology.

Current histopathological classification

As described by the World Health Organization (WHO) classification (Louis et al. 2007), malignant diffuse gliomas are comprised of astrocytic, oligodengroglial, and mixed oligoastrocytic neoplasms based solely on morphology and are further subdivided by tumor grade based on additional histologic features present in the tumor. Nuclear atypia and mitotic activity are required criteria for grade III lesions, and the presence of necrosis or microvascular proliferation is required for the diagnosis of grade IV astrocytoma, glioblastoma (Miller and Perry 2007). While 90%–95% of glioblastomas arise de novo and are considered "primary," ~5%–10% arise from lower-grade gliomas in younger patients and are termed "secondary" (Biernat et al. 2004; Ohgaki and Kleihues 2005). Together with grade III anaplastic astrocytoma, which has an incidence of >1500 cases annually (CBTRUS 2011), these tumors comprise the clinical entity termed "malignant glioma." However, as discussed below, emerging classification schemes are likely to incorporate molecular differences that distinguish these tumors (von Deimling et al. 2011).

Large-scale genomic studies of glial neoplasms

Glioblastoma, like other cancers, is the product of accumulated genetic and epigenetic alterations (Vogelstein and Kinzler 2004; Weir et al. 2004; Stratton et al. 2009), and the application of genome-scale approaches to enumerate these genetic alterations has uncovered both molecular subclasses and common pathways mutated in this disease. To date, glioblastoma has been subjected to the most extensive genomic profiling of any cancer, and thus we review here our current understanding of the critical underlying molecular pathology in this disease that has come from an integrated view of somatic copy number alterations (SCNAs) and sequence mutations and also review recent discoveries in lower-grade astrocytomas.

Integrated approaches to characterize the genomic landscape of glioblastoma

Malignant gliomas, like other human cancers, are characterized by genetic instability and complex alterations in chromosome structure and copy number. The SCNAs found in malignant gliomas include broad or regional alterations spanning segments or whole arms of entire chromosomes as well as focal events involving one or a few genes. The development of high-resolution, array-based comparative genomic hybridization (CGH) using bacterial artificial chromosomes, oligomers, and single-nucleotide polymorphism (SNP) arrays made possible the systematic analysis of cancer genomes and defined many new recurrent SCNAs in cancer (Chin et al. 2011). To fully explore these data, several groups have also developed bioinformatic tools to highlight the cancer-derived genomic alteration signals from among the background noise (Beroukhim et al. 2007; Greenman et al. 2007; Wiedemeyer et al. 2008; Riddick and Fine 2011). These methodologies, including Genomic Identification of Significant Targets in Cancer (GISTIC) (Beroukhim et al. 2007; Mermel et al. 2011) and Genomic Topography Scan (GTS) (Wiedemeyer et al. 2008), were first deployed in glioblastoma and subsequently across thousands of cancer samples (Beroukhim et al. 2010; http://www.broadinstitute.org/tumorscape). Combined work using these tools and others (Bredel et al. 2005; Kotliarov et al. 2006) identified overlapping presumed targets of amplification (including *EGFR*, *MET*, *PDGFRA*, *MDM4*, *MDM2*, *CCND2*, *PIK3CA*, *MYC*, *CDK4*, and *CDK6*) and deletion (including *CDKN2A/B*, *CDKN2C*, *PTEN*, and *RB1*) in glioblastoma.

This work in SCNAs preceded two recent studies that further clarified our understanding of the glioblastoma genomic landscape (The Cancer Genome Atlas Research Network 2008; Parsons et al. 2008). The Cancer Genome Atlas (TCGA) pilot project, the first of many ongoing comprehensive TCGA consortium-based cancer genome analyses (http://cancergenome.nih.gov), applied multiplatform profiling to systematically and comprehensively define the genomic landscape of glioblastoma. This approach used targeted Sanger sequencing to investigate 601 genes in 91 samples as well as array-based platforms to analyze copy number, mRNA expression, and the epigenetic state of ~200 tumors, most of which were untreated, primary glioblastomas. This larger effort confirmed prior SCNA analyses on smaller numbers of tumors and also identified novel lesions, such as homozygous deletions of *PARK2* and amplification of *AKT3*. Moreover, separate work also showed that NF-κB inhibitor α (*NFKBIA*) is heterozygously deleted in a mutually exclusive manner to *EGFR* amplification (Bredel et al. 2011).

The most significantly somatically mutated genes (false discovery rate <0.1) among the 601 genes analyzed were *TP53* (42%), *PTEN* (33%), *neurofibromatosis-1* (*NF1*) (21%), *EGFR* (18%), *RB1* (11%), *PIK3R1* (10%), and *PIK3CA* (7%). However, this effort demonstrated that the integration of multiple types of genomic analysis permitted the projection of identified alterations onto known pathways (Fig. 1). This approach revealed the high incidence of p53, Rb, and receptor tyrosine kinase (RTK)/Ras/phosphoinositide 3-kinase (PI3K) pathway dysregulation, confirming previous work that had delineated lesions in these critical cascades (Ekstrand et al. 1991; Henson et al. 1994; Louis 1994, 2006; Reifenberger et al. 1994; Schmidt et al. 1994; Ueki et al. 1996). Specifically, p53 signaling was impaired in 87% of the samples through *CDKN2A* deletion (49%), *MDM2* (14%) and

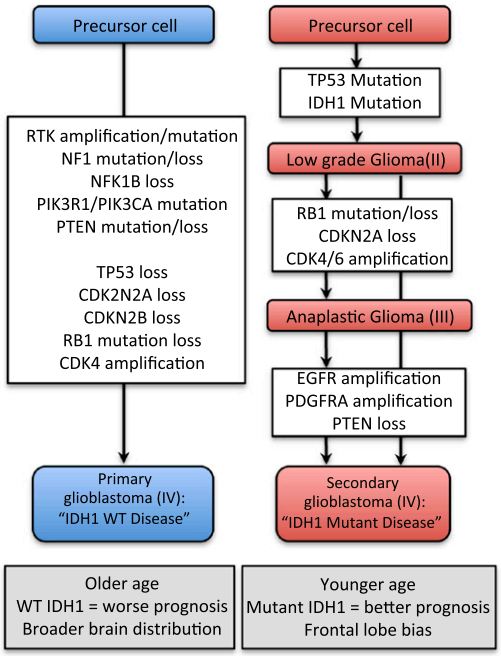


Figure 1. Genomic alterations underlying gliomagenesis. Both primary and secondary glioblastomas arise from precursor cells that may be distinct. Primary glioblastomas arise de novo and exhibit p53 and Rb pathway dysfunction as well as RTK/Ras/PI3K signaling dysregulation, leading to tumors that arise in older patients with a worse prognosis, likely owing to the predominant wild-type *IDH1* genotype. In contrast, secondary glioblastomas are preceded by lower-grade II lesions, which progress either through grade III lesions or directly to glioblastoma. These tumors occur in younger patients and are dominated by a mutant *IDH1* genotype that confers a better prognosis and is associated with a more restricted frontal lobe location.

MDM4 (7%) amplification, and mutation and deletion of *TP53* (35%). Likewise, Rb signaling was impaired in 78% of the samples through *CDKN2* family deletion; amplification of *CDK4* (18%), *CDK6* (1%), and *CCND2* (2%); and mutation or deletion of *RB1* (11%). Additional work also showed that CDK6 is an oncogene, and particular patterns of *CDKN2* loss can predict cellular dependency on CDK4 and CDK6 inhibition (Wiedemeyer et al. 2010). Finally, evidence of RTK/RAS/PI3K activation was found in 88% of tumors, including contributions from unexpected mutations or deletions in *NF1* (18%) and *PIK3R1*, which encodes the p85α regulatory subunit of PIK3CA. Building on this pilot glioblastoma effort, TCGA is profiling primary glioblastoma samples with the goal of achieving complete genomic characterization of >500 tumors. As next-generation sequencing technologies (Meyerson et al. 2010) are incorporated into the TCGA pipeline, many of these glioblastoma specimens will undergo whole-exome, transcriptome, and/or whole-genome sequencing, providing higher-resolution detail of lower-frequency somatic alterations, including intrachromosomal and interchromosomal translocations. A subset of these data from nearly 500 tumors is available through the TCGA portal (http://tcga-data.nci.nih.gov/tcga).

In parallel to TCGA, the Vogelstein laboratory (Parsons et al. 2008) pursued a complementary strategy to characterize glioblastoma genomes composed of sequence, copy number, and expression analysis of a greater number of genes in fewer tumors—specifically, 23,219 transcripts from 20,661 protein-coding genes in 22 malignant gliomas were analyzed by Sanger-based sequencing. Strikingly, five out of 22 tumors, which included one "high-grade" glioma and one secondary glioblastoma, harbored recurrent R132H-encoded substitutions in the isocitrate dehydrogenase 1 (*IDH1*) gene. These findings were extended to 18 out of 149 malignant gliomas, which included mostly primary glioblastomas but also a proportion of secondary tumors. Three IDH isoforms exist in humans: IDH1 is a cytosolic protein, whereas IDH2 and IDH3 are located within the mitochondria. IDH1 and IDH2 are known to catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG), leading to the production of NADPH, in the tricarboxylic acid (TCA) cycle, a biochemical sequence critical in sugar, lipid, and amino acid metabolism (Raimundo et al. 2011). Although a missense mutation in *IDH1*, encoding IDH1 R132C, was first identified in one patient with colon cancer in a sequencing analysis of the coding regions in breast and colon cancer (Sjoblom et al. 2006), this study provided the first evidence of recurrent mutations in *IDH1*.

Subsequent work from several groups has provided a comprehensive picture of the IDH status in brain tumors (Balss et al. 2008; Hartmann et al. 2009; Ichimura et al. 2009; Yan et al. 2009). *IDH1/2* is mutated in grade II and III gliomas as well the secondary glioblastomas that arise from prior low-grade tumors, with most mutations found in the *IDH1* gene. Importantly, these mutations usually occur at conserved residues and are virtually never homozygous. Specifically, whereas only 3%–7% of primary glioblastomas harbor *IDH1* mutations, the majority (50%–80%) of secondary glioblastomas express mutant IDH1. Furthermore, most lower-grade gliomas harbor *IDH1* mutations; although grade I pilocytic astrocytomas usually express wild-type IDH1, ~60%–80% of grade II and III astrocytomas, oligodendrogliomas, and oligoastrocytomas express mutant IDH1, with the R132H mutation representing the majority of mutations observed. In addition, ~3% of these tumors that express wild-type IDH1 were found to express IDH2 R172 mutations (Balss et al. 2008; Hartmann et al. 2009; Ichimura et al. 2009; Yan et al. 2009), although this mutation in *IDH2* has only been documented in a single glioblastoma in the literature (Hartmann et al. 2010). Other CNS tumors found to harbor *IDH1* mutations include gangliogliomas, giant cell glioblastomas, and primitive neuroectodermal tumors, although small numbers of these tumors have been studied (Balss et al. 2008). Whereas mutations in other TCA cycle enzymes, such as fumarate hydratase in leiomyomas and renal cell cancer and succinate dehydrogenase in paragangliomas, have been identified (Kaelin 2011; Raimundo et al. 2011), mutations in these genes have not been found in gliomas.

IDH1/2 mutations have also been identified in 12%–17% of acute myeloid leukemias (AMLs) (Mardis et al. 2009; Paschka et al. 2010; Ward et al. 2010; Graubert and

Mardis 2011), the majority of central and periosteal cartilaginous tumors (Amary et al. 2011a), and also 23% of cholangiocarcinomas (Borger et al. 2012). Interestingly, somatic mosaic *IDH1/2* mutations were found to be the likely genetic basis of Ollier disease and Maffuci syndrome (Amary et al. 2011b; Pansuriya et al. 2011). These rare, nonfamilial conditions, both characterized by the early development of multiple cartilaginous tumors, have also been reported to manifest concomitant glioma or AML, thereby providing an intriguing demonstration of the likely causal role that mutant IDH1/2 plays in these three distinct tumor types (Rawlings et al. 1987). Finally, 50% of patients with D-2-hydroxyglutaric aciduria (D-2-HGA), a rare inherited neurometabolic disorder, have been found to carry *IDH2* mutations (Kranendijk et al. 2010). Indeed, the discovery of *IDH1/2* mutations is one of the major novel findings to emerge from genome annotation studies and has stimulated renewed attention to altered metabolism in cancer biology.

The genetic basis of oligodendrogliomas and pediatric gliomas

In addition to the frequency of *IDH1* mutations in grade II glioma, cancer sequencing studies have provided new insights into the genetic basis of other lower-grade glial neoplasms. Specifically, >50% of oligodendrogliomas display loss of heterozygosity (LOH) at chromosomes 1p and 19q (Cairncross et al. 1998), although the targets of these deletions have remained elusive. However, Bettegowda et al. (2011) recently used next-generation sequencing to analyze the exomes of seven anaplastic oligodendrogliomas (WHO grade III) and found novel recurrent inactivating mutations affecting *FUBP1* (far-upstream element [FUSE]-binding protein 1; five out of 34 tumors), a regulator of MYC signaling located on chromosome 1p, and the homolog of *Drosopila capicua*, *CIC* (18 out of 34 tumors), a downstream transcriptional repressor of RTK/MAPK signaling located on chromosome 19q. Yip et al. (2012) confirmed the high incidence of *CIC* mutation with concurrent 1p/19q loss and *IDH1* mutation in their series.

Recent work has also identified a high incidence of specific mutations in two types of pediatric gliomas. First, several studies have revealed *BRAF* alterations in lower-grade pediatric tumors. Copy number analysis of WHO grade I pilocytic astroctyomas identified a tandem duplication at chromosome 7q34 resulting in a novel oncogenic *BRAF* fusion gene, *KIAA1549:BRAF*, in >60% of these tumors (Bar et al. 2008; Jones et al. 2008; Pfister et al. 2008). Together with other identified fusion events such as *SRGAP3:RAF1* (Jones et al. 2009), *RAF* fusion events occur in >80% of pilocytic astrocytomas (von Deimling et al. 2011). Furthermore, $BRAF^{V600E}$ mutations have been found most commonly in WHO grade II pleomorphic xanthoastrocytomas (66%) (Dias-Santagata et al. 2011; Schindler et al. 2011) as well as WHO grade I gangliogliomas (18%) (MacConaill et al. 2009; Schindler et al. 2011). In addition, Wu et al. (2012) used whole-genome sequencing to identify recurrent mutations in *H3FA*, which encodes the H3.3 protein, and the closely related *HIST1H3B* gene, which encodes the H3.1 protein isoform, in pediatric diffuse pontine gliomas. Mutations in these two genes were found in 78% of these tumors, 22% of nonbrainstem pediatric glioblastomas, and virtually no other CNS tumors evaluated. Together, these findings have clear implications for taxonomic classification and, in the case of *BRAF* alterations, potential targeted therapies.

Transcriptional profiling: identification of subtypes and biological programs in malignant glioma

Classification

The genome-wide analysis of mRNA expression to identify molecular subclasses (Golub et al. 1999) has led to a fundamental shift in our understanding of glioblastoma subtypes. Indeed, the identification of multiple subtypes within glioblastoma has underscored the heterogeneity of diseases that all share the same WHO histopathological grade. This approach has revealed that the glioma transcriptome is highly structured and reflects tumor histology, molecular alterations, and clinical outcome (Nutt et al. 2003; Freije et al. 2004; Phillips et al. 2006; Verhaak et al. 2010; Brennan 2011; Huse et al. 2011).

Although expression profiling of glioblastoma has been used by many groups (for review, see Brennan 2011; Huse et al. 2011), two studies have provided the foundation for classification of glioblastoma subtypes (Phillips et al. 2006; Verhaak et al. 2010). Using unsupervised hierarchical clustering analysis, Verhaak et al. (2010) classifed 200 TCGA glioblastoma samples into four subtypes (Table 1), which were subsequently validated using previously published data from 260 independent samples. Each of the four subtypes was ultimately defined by a minimum list of 210 genes (http://tcga-data.nci.nih.gov/docs/publications/gbm_exp). By incorporating the available copy number and sequence data, three of the four subtypes were found to harbor distinct molecular alterations. Specifically, the proneural subtype was enriched for amplifications of *PDGFRA*, *CDK6*, *CDK4*, and *MET*; 11 out of 12 *IDH1* mutations found in the TCGA samples; *PIK3CA/PIK3R1* mutations; and mutation or LOH of *TP53*. Of note, this subtype contained the highest percentage of young patients, likely due in part to the high number of *IDH1* mutant tumors in this category. The classical subtype was enriched for amplification of *EGFR* and loss of *PTEN* and *CDKN2A*, whereas the mesenchymal subtype harbored mutations and/or loss of *NF1*, *TP53*, and *CDKN2A*. To date, no unique genetic alterations define the neural class from the other classes.

In contrast to Verhaak et al. (2010), Phillips et al. (2006) identified three distinct glioblastoma subtypes based on their differences of expression of a panel of genes most strongly correlated with survival. This approach delineated subtypes that were termed proneural, mesenchymal, and proliferative. Although the number of subtypes identified by the Verhaak et al. (2010) and Phillips et al. (2006) studies differs, the proneural and mesenchymal classifications identified using distinct methodologies

Table 1. *Molecular heterogeneity and classification in gliomas/astrocytomas*

	Primary glioblastoma (IV)				Secondary glioblastoma (IV)	Glioma (II–III)	Oligodendroglioma (II–III)	DIPG (III–IV) (pediatric)	PA, PXA(I) (pediatric)
Transcriptional subtype	Mesenchymal	Classical	Neural	Proneural					
IDH1/2 status	Wild type	Wild type	Wild type	Mutant	Mutant	Mutant	Mutant		
Methylation feature				G-CIMP$^+$	G-CIMP$^+$	G-CIMP$^+$	G-CIMP$^+$		
Signature mutations	NF1			TP53	TP53	TP53	CIC	H3F3A, HIST1H3B	$BRAF^{V600E}$ (PXA), BRAF fusions (PA)
Signature CNAs	NF1 loss	PTEN loss EGFR amp		PDGFRA amp MET amp	PDGFRA amp		1p/19q loss		
Proteomic features	NF1 signaling, YKL-40, VEGF, CD44, IRS1	EGFR signaling, Notch signaling		PDGFB signaling					
Transcriptional regulators	STAT3, CEBP/β, TAZ								

Primary glioblastomas are characterized by particular transcriptomic subtypes that are further described by specific genomic and proteomic features. Secondary glioblastomas as well as lower-grade gliomas also exhibit signature genomic alterations.
(PA) Pilocytic astrocytoma; (PXA) pilocytic xanthoastrocytoma; (DIPG) diffuse intrinsinc pontine glioma (DIPG).

and sample sets are the most robust and concordant (Huse et al. 2011); genes defining the Verhaak and Phillips subclasses are listed together in Supplemental Table S1B of Bhat et al. (2011,). For instance, both groups identified proneural class expression of *DLL3* and *OLIG2* and mesenchymal class expression of *CD40* and *CHI3L1/YKL-40*, the latter of which appears to be a potential serum protein marker of prognosis in glioblastoma patients (Iwamoto et al. 2011). Moreover, a subset of the genes represented in these subtypes is represented in a nine-gene panel shown to predict outcome in glioblastoma, as increased expression of mesenchymal genes such as *CHI3L1/YKL-40* and *LGALS3* combined with decreased expression of a proneural gene, *OLIG2*, were associated with typical short-term survival compared with longer-term survivors (Colman et al. 2010). Considering that other groups have used alternative methodologies to derive nonoverlapping prognostically significant gene sets (Bredel et al. 2009), further work in large, relatively uniform sample sets will be necessary to resolve subtype classification systems and validate multiple prognostic gene sets.

To determine whether these genomically identified subtypes predict biological or clinical differences in glioblastoma, Brennan et al. (2009) used a targeted proteomics approach to determine whether glioblastomas also segregated into distinct classes by activation of signal transduction pathways. Unsupervised clustering of 57 proteins or protein modifications assessed in 20 glioblastoma samples identified distinct tumor subgroups defined by EGFR-related signaling, PDGF-related signaling, or proteins associated with decreased NF1 expression. Moreover, analysis of TCGA glioblastoma expression profiles showed that SCNAs or mutations of *EGFR*, *PDGFRA*, and *NF1* were largely mutually exclusive, suggesting that the identified signaling nodes are nonoverlapping. Interestingly, because PDGF core tumors often expressed high PDGF protein but low PDGF mRNA and no *PDGFRA* SCNAs, it is possible that PDGF/PDGFRA-mediated biology is underestimated in purely mRNA-based expression schemes. Moreover, the finding that EGFR core tumors harbored evidence of active Notch signaling not reflected in copy number, expression, or sequence data was additional evidence that not all biologically important information can be gleaned from genomic data. This study supports the integration of protein-based biomarker assessment, perhaps by immunohistochemistry for a targeted biomarker panel, into the broader subtype classification architecture currently defined by genomic profiling.

Network analysis of expression profiles

Additional work has extended the utility of mRNA profiling by using computational network analysis to uncover the causal regulatory modules underlying particular transcriptomically defined subtypes. Broadly, these approaches attempt to infer the upstream biological programs, or networks, that coordinately produce a given transcriptional state. Recently, two groups used a reverse engineering-based algorithm called ARACNe (algorithm for the reconstruction of accurate cellular networks) (Basso et al. 2005) to identify transcription factors that regulate the mesenchymal expression state (Carro et al. 2010; Bhat et al. 2011). Using ARACNe and a novel master regulatory algorithm, Carro et al. (2010) identified six transcription factors—STAT3, C/EBPβ, RUNX1, FOSL, bHLH-B2, and ZNF-238—that are likely to control the majority of mesenchymal subtype tumors. Overexpression of STAT3 and C/EBPβ both induced a mesenchymal phenotype and were individually required for orthotopic xenograft growth. Bhat et al. (2011) also applied the ARACNe method to TCGA samples to identify transcription factors that regulated the mesenchymal subtype and identified several distinct components, including TAZ, YAP, MAFB, and HCLS1. Follow-up work demonstrated a pivotal involvement of TAZ and dysregulated Hippo pathway signaling in driving mesenchymal biology; TAZ tended to be hypermethylated in proneural tumors, could drive a mesenchymal phenotype when overexpressed, and cooperated with PDGF-B to induce malignant mesenchymal-type gliomas in the RCAS/N-*tva* mouse model. Together, these network analyses of gene expression data demonstrate the utility of these data sets not only to classify distinct tumor states, but also to provide clues to the underlying biology.

Signature molecular lesions in glioma: key drivers, tumor suppressors, and the IDH proteins

Combined work from genomic and proteomic analyses has focused attention on critical pathways—driven by the dysregulation of driver and checkpoint proteins—that contribute to gliomagenesis (Phillips et al. 2006; The Cancer Genome Atlas Research Network 2008; Parsons et al. 2008; Brennan et al. 2009; Verhaak et al. 2010). In particular, it is clear that RTKs (including EGFR, PDGFRA, and MET), the PI3K pathway, signaling pathways activated by *PTEN* and *NF1* loss, and the mutant IDH proteins play central roles in the pathobiology of glioblastoma.

EGFR/EGFRvIII

EGFR amplification is observed in ~50% of primary glioblastomas and is associated with poor prognosis (Hurtt et al. 1992; Jaros et al. 1992; Schlegel et al. 1994). Furthermore, ~50% of *EGFR*-amplified cells harbor the *EGFRvIII* mutant, which is an intragenic gene rearrangement generated by an in-frame deletion of exons 2–7 that encode part of the extracellular region. The expression of EGFRvIII has been determined to confer a worse prognosis than wild-type EGFR expression alone (Shinojima et al. 2003; Heimberger et al. 2005). Experimentally, ectopic overexpression of EGFRvIII in glioma cell lines induces constitutive autophosphorylation, activation of the Shc–Grb2–Ras and class I PI3K pathways (Huang et al. 1997; Narita et al. 2002), enhanced tumorigenicity (Huang et al. 1997), increased cell proliferation (Narita et al. 2002), and resistance to apoptosis induced by DNA-damaging agents through modulation of Bcl-X_L expression (Nagane et al. 1998). Notably, the downstream effects of EGFRvIII

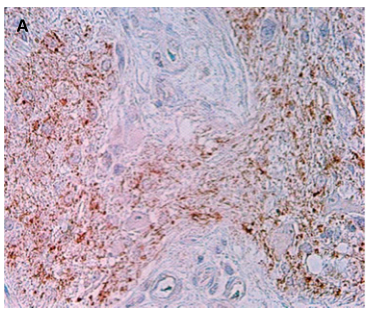
overexpresson are not recapitulated by overexpression of wild-type EGFR. For example, wild-type EGFR cannot substitute for EGFRvIII in driving infiltrative glioma formation in genetically engineered mice (Hesselager and Holland 2003; Zhu et al. 2009) or in *Ink4a/Arf*$^{-/-}$ murine neural stem cells or astrocytes (Holland et al. 1998; Bachoo et al. 2002), except when EGF ligand is infused at a high concentration into the injection site of wild-type EGFR-transduced cells (Bachoo et al. 2002). Both EGFRvIII and wild-type EGFR/ErbB family proteins have been identified in the nucleus and are thought to drive proliferation and DNA damage repair through both transcriptional and signaling functions (Wang and Hung 2009). Moreover, the observation that EGFR also translocates to the mitochondria (Boerner et al. 2004) provides further evidence that the contributions of EGFR malignancy may not be limited to its conventional cell membrane location and merit further study.

Despite the well-recognized proproliferative functions of EGFRvIII, its expression in human glioblastoma is heterogeneous and is most often observed only in a subpopulation of cells (Fig. 2A; Nishikawa et al. 2004). Recent observations support a model of functional heterogeneity in which a minority of EGFRvIII-expressing cells not only drive their own intrinsic growth, but also potentiate the proliferation of adjacent wild-type EGFR-expressing cells in a paracrine fashion through the cytokine coreceptor gp130 (Inda et al. 2010). Even though these results illustrate that cytokines produced from EGFRvIII expression can be drivers of heterogeneity, there are likely additional cytokine-inducing mechanisms at work. For example, it has been recently shown that *NFKB1A*, which encodes IκBα, a critical negative regulator of canonical NF-κB activation, was found to undergo monoallelic loss in glioblastomas that lack *EGFR* amplification (Bredel et al. 2011), suggesting that NF-κB plays physiologically relevant roles downstream from EGFR/EGFRvIII that include IL-8 production (Bonavia et al. 2011). These results suggest that intraclonal cooperativity drives the persistence of intratumoral heterogeneity, which has implications for both our basic understanding of gliomagenesis and also drug sensitivity profiles of these tumors (Yao et al. 2010).

In addition, although most human glioma cell lines fail to faithfully recapitulate the *EGFR* amplification and EGFRvIII expression observed in primary tumor specimens, recent studies have reported successful passage of EGFRvIII-expressing glioblastoma xenografts in vivo as well as in vitro when grown in stem cell culture conditions (Stockhausen et al. 2011), suggesting that durable EGFRvIII expression may be linked to differentiation and/or development. Moreover, it is now clear that the type of genetic alterations involving EGFR in glioblastoma are distinct from those observed in other EGFR-altered cancers, such as non-small-cell lung cancer (NSCLC). In glioma, focal EGFR amplification occurs at an extremely high level (>20 copies). In addition, the vast majority of other mutations, including the *vIII* mutant as well as missense mutations (Lee et al. 2006b; The Cancer Genome Atlas Research Network 2008), are found within the extracellular domain, while most mutations in other nonglioma cancers are found in the intracellular domain (Janne et al. 2005). Although EGFRvIII expression is sufficient to rescue the knockdown of an endogenous kinase domain mutant EGFR in NSCLC cells (Rothenberg et al. 2008), it is not clear whether EGFR mutant glioma cells drive similar downstream signaling and/or confer the same "addiction" to EGFR activation as is the case in NSCLC (Sharma and Settleman 2007).

PDGFR

Nearly 30% of human gliomas show expression patterns that are correlated with PDGFR signaling (Brennan et al. 2009) and genes involved in oligodendrocyte development (*OLIG2*, *NKX2-2*, and *PDGF*), both hallmarks of the proneural glioblastoma subtype. *PDGFRA* amplification is found in 15% of all tumors and is enriched in the proneural subtype (Phillips et al. 2006; Verhaak et al. 2010). Of those tumors harboring gene amplification, recent work showed that 40% harbor an intragenic deletion, termed *PDGFRA*$^{\Delta 8,9}$ (Clarke and Dirks 2003), in which an in-frame deletion of 243 base pairs (bp) of exons 8 and 9 leads to a truncated extracellular domain (Ozawa et al. 2010). In addition, in-frame gene fusion of the extracellular domain of *KDR/VEGFR-2* and the kinase and intracellular domains of *PDGFRA* has also been identified, and both the PDGFRA$^{\Delta 8,9}$ and KDR-PDGFRA mutant proteins were constitutively active and transforming and could be inhibited with inhibitors of PDGFRA. Point mutations in *PDGFRA* are associated with amplification but, unlike *EGFR*, are generally rare events (The Cancer Genome Atlas Research Network 2008). Of the additional ways to activate PDGFR signaling, PDGF ligands (A–D) are up-regulated in ~30% of glioma surgical



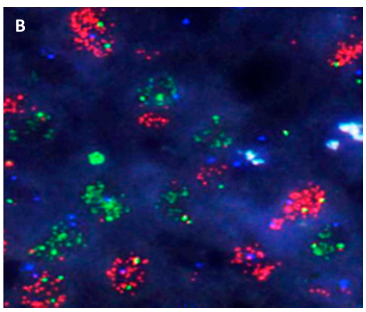


Figure 2. Molecular heterogeneity in glioblastoma. (*A*) Immunohistochemistry for the mutant EGFR receptor EGFRvIII demonstrates a heterogeneous staining pattern within the tumor. Images from Nishikawa et al. (2004) used with permission. (*B*) Multicolor FISH reveals distinct subpopulations of either *EGFR* (red) or *PDGFRA* (green) amplification within a glioblastoma specimen. Images obtained from Cameron Brennan.

samples and cell lines, while PDGFRB expression appears to be restricted to proliferating endothelial cells in glioblastoma (Fleming et al. 1992; Hermanson et al. 1992; Di Rocco et al. 1998; Smith et al. 2000; Lokker et al. 2002). The intratumoral coexpression of PDGF and PDGFR suggests that both autocrine and paracrine loops play roles in glioblastoma. This possibility is supported by the demonstration that stimulation of PDGFRB-expressing endothelial cells by tumor-derived PDGF can drive VEGF-mediated angiogenesis within the local microenvironment (Guo et al. 2003).

Similar to the case of EGFR/EGFRvIII described above, cell and receptor heterogeneities appear to be other distinct mechanisms by which PDGF/PDGFR promotes aggressive glioma growth. For example, transduction of cells of the subventricular zone (SVZ) of the lateral ventricle of neonatal rat pups with a retrovirus expressing PDGF yielded large, diffusely infiltrating tumors resembling glioblastoma (Assanah et al. 2006, 2009). The tumors that formed contained a massive proliferation of both infected and uninfected PDGFRα^+-expressing progenitors, suggesting that PDGF was driving tumor formation through both autocrine and paracrine signaling, leading to the recruitment of non-PDGF-expressing resident progenitors and the evolution of cellularly heterogeneous malignant gliomas. Further work has demonstrated that these recruited cells become transformed, overtake PDGF-induced gliomas, and can be serially transplanted (Fomchenko et al. 2011), and that topotecan delivery to these tumors results in ablation of both tumor-initiating cells and recruited glial progenitors (Lopez et al. 2011). Although these results still remain to be validated within the context of human tumors, they raise the possibility that cells distinct from the initial transformed cells of origin within the tumor environment can be corrupted to become bona fide tumor cells. This model of glioma evolution is distinct from the generally established view of linear gliomagenesis (Fomchenko et al. 2011).

c-Met

The c-Met RTK is amplified in ~5% of glioblastomas, has been found to be overexpressed in 18 out of 62 (29%) glioblastoma samples with shorter median survival (Kong et al. 2009), and is rarely mutated (The Cancer Genome Atlas Research Network 2008). Moreover, c-MET was found to be coactivated in glioblastoma cells with increased levels of EGFR/EGFRvIII (Huang et al. 2007; Stommel et al. 2007; Pillay et al. 2009) and represents a critical dependency in *MET*-amplified cells (Beroukhim et al. 2007). In this setting, activated EGFR can associate with c-Met, leading to the activation of this receptor in the absence of its ligand, HGF (Jo et al. 2000). Conversely, HGF transcriptionally activates the expression of the EGFR ligands TGF-α- and heparin-binding EGF and can therefore activate EGFR (Reznik et al. 2008). Blocking EGFRvIII activity with an EGFR-specific monoclonal antibody, panitumumab, can result in a switch to HGF-mediated c-Met activation, which could be prevented by cotreatment with AMG102, a neutralizing antibody to HGF (Pillay et al. 2009). Similar results were achieved with combined c-Met and EGFR small molecule inhibitors (Huang et al. 2007; Stommel et al. 2007) and neutralizing anti-HGF monoclonal antibody combined with erlotinib (Lal et al. 2009). The relationship of c-Met with EGFR is also not surprising in light of prior studies showing that overexpression of Met can lead to gefitinib resistance in mutant EGFR lung cancers, often by activating ERBB3 (Engelman et al. 2007). Concurrent activation of c-Met with PDGFR has also been detected in glioblastoma and has been suspected to be another mechanism for resistance to EGFR kinase inhibitors. In this case, inhibition of three RTKS—EGFR inhibition with erlotinib, c-Met inhibition with SU11274, and PDGFR inhibition with imatinib—significantly inhibited the in vitro growth of glioblastoma cell lines, compared with single drugs alone (Stommel et al. 2007), possibly by attenuating downstream PI3K signaling. In contrast, in scenarios in which *MET* is amplified in isolation, one report showed that treatment with crizotinib, which inhibits ALK and also c-Met (Christensen et al. 2007), can induce radiographic and clinical improvement (Chi et al. 2012).

Src family kinases (SFKs)

SRC and SFKs are frequently activated in glioblastoma patient samples and cell lines (Stettner et al. 2005; Du et al. 2009) and are widely expressed in glioblastoma (Lu et al. 2009). SFKs mediate signaling from growth factor receptors that are commonly overexpressed in glioblastoma, providing a potential explanation for SFK activation. Bead-based profiling of tyrosine kinase activation in 130 human cancer cells showed that the most frequently activated tyrosine kinases were EGFR, fibroblast growth factor receptor 3 (FGFR3), protein tyrosine kinase 2 (PTK2, also known as focal adhesion kinase, or FAK), and SFKs including SRC, LYN, and LCK (Du et al. 2009). Moreover, screening of 31 primary glioblastomas samples showed SRC activation in 61% of samples (Du et al. 2009) and that the SRC inhibitor dasatinib inhibited cell viability and migration in vitro and tumor growth in vivo, nominating SRC/SFK as potential therapeutic targets in a subset of glioblastomas.

RTK cooperativity and heterogeneity

One potential explanation for the failure of EGFR and PDGFRA inhibitors to elicit significant clinical outcomes (De Witt Hamer 2010) is that additional RTKs may cooperate to provide an integrated signaling threshold that is not sufficiently attenuated through the inactivation of any single RTK (Huang et al. 2007; Stommel et al. 2007). Indeed, Stommel et al. (2007) demonstrated that three or more RTKs were activated in a majority of glioblastoma cell lines and patient specimens. This discovery of concomitant receptor expression and coactivation suggests that tumor RTK profiling may be an important step in the development of a personalized glioblastoma therapeutic regimen and that cross-talk between the receptors could be targeted with specific inhibitors to both, resulting in enhanced cytotoxicity.

Recent studies provide additional evidence that glioblastomas are composed of heterogeneous subpopulations within tumors. Indeed, varied expression patterns of several proteins have been described: Wild-type EGFR and EGFRvIII, as described above (Nishikawa et al. 2004; Inda et al. 2010); PDGFRA (Hermanson et al. 1992); c-Met (Nabeshima et al. 1997); angiogenic factors (Koga et al. 2001); and adhesion molecules (Bello et al. 2001b) all exhibit heterogeneous distributions. Two recent studies provide further compelling examples of clonal heterogeneity in glioblastoma. Both Snuderl et al. (2011) and Szerlip et al. (2012) observed that 5%–7% of all glioblastomas—and nearly 13% of glioblastomas with *EGFR*, *PDGFRA*, or *MET* amplification—harbored multiple RTK amplifications. Moreover, while a minority of cells harbored amplification of multiple RTKs (Szerlip et al. 2012), the predominant pattern was mosaic amplification such that tumors were comprised of individual cell populations harboring isolated amplification of a single RTK (Fig. 2B). This point was further demonstrated in a remarkable FISH analysis of tumor sections from a whole-brain autopsy of an untreated patient with bilateral multifocal glioblastoma in which there was a striking anatomic distribution of *EGFR*- and *PDGFRA*-amplified cells (Snuderl et al. 2011). It is likely that this pattern of clonal RTK heterogeneity is a late event in gliomagenesis (Snuderl et al. 2011; Szerlip et al. 2012) and is influenced by several factors, including binomial segregation of unstable amplicons or local microenvironment selection (Szerlip et al. 2012). It is likely that somatic mutations in cancer genes will demonstrate a pattern of heterogeneity similar to that seen with SCNAs, but this possibility awaits additional study at this point. Together, these studies provide further molecular evidence that glioblastomas are often heterogeneous, which has major biologic and therapeutic implications.

PI3K pathway

Activating kinases The PI3K signaling pathway is dysregulated in many cancers (Yuan and Cantley 2008), including glioblastomas. Some of the major genomic alterations discussed above—RTK amplification/mutation, *PIK3CA* and *PIK3R1* mutation, and *PTEN* loss—all activate this pathway (The Cancer Genome Atlas Research Network 2008). Of the three PI3K classes, the class IA kinases are likely to play a direct role in cell transformation and are composed of both a catalytic subunit isoform (p110α, p110β, p110δ, and p110γ) and a regulatory subunit isoform (p85α, p55α, p50α, p85β, and p55γ). Broadly, activation of the pathway can be initiated by GTP-bound Ras (Rodriguez-Viciana et al. 1994, 1996) and through RTK signaling, which recruits PI3K to the cell membrane, whereupon the lipid phosphatidylinositol (PtdIns)-4,5-bisphosphate (PIP2) is phosphorylated to PtdIns-3,4,5-bisphosphate (PIP3). This reaction is antagonized by the major glioma tumor suppressor and PtdIns(3,4,5)P_3 phosphatase PTEN. PIP3 subsequently recruits the serine/threonine kinase AKT to the plasma membrane, where it is fully activated by its main dual inputs—Thr 308 phosphorylation by PDK1, and Ser 473 phosphorylation by the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) (Sarbassov et al. 2005). Elevated AKT phosphorylation has been observed in up to 85% of glioblastoma cell lines and patient samples (Wang et al. 2004). RTK-independent activation of this pathway in glioblastoma can occur via mutation or amplification of *PIK3CA* (*p110α*) (Gallia et al. 2006; Kita et al. 2007; The Cancer Genome Atlas Research Network 2008), and PIK3CD (p110δ) is also overexpressed in some gliomas (Mizoguchi et al. 2004). Moreover, the TCGA study revealed recurrent mutations in the gene encoding the p85α regulatory subunit *PIK3R1*, which likely drive PIK3CA activation through decreased SH2 domain-mediated inhibition (Sun et al. 2010).

PI3K drives many glioma-relevant processes, including survival, proliferation, migration, and invasion (Engelman et al. 2006). In addition to its well-known functions (Lino and Merlo 2011), recent work has revealed several novel mechanisms underlying PI3K function in glioma. Specifically, CD95 promotes invasion through interaction with PI3K and the SFK Yes, triggering activation of GSK3β and induction of matrix metalloproteinase (MMP) expression (Kleber et al. 2008). The RTK EphA2, which is highly expressed in glioblastoma (Wykosky et al. 2005) and binds PI3K upon ligand stimulation (Pandey et al. 1994), induces glioblastoma cell migration in an AKT-dependent manner (Miao et al. 2009). In addition, the insulin-like growth factor 2 (IGF2) promotes aggressive growth in glioblastoma lacking EGFR amplification or overexpression through IGF receptor 1 (IGFR1) and PIK3R3 (Soroceanu et al. 2007). The PI3K pathway also maintains glioblastoma tumor-initiating cells, as direct silencing of mTOR or inactivation with rapamycin reduces neurosphere formation and expression of neural stem cell progenitor markers (Sunayama et al. 2010) as well as growth of patient-derived tumor-initiating cells (Gallia et al. 2009). In addition, AKT activation due to *PTEN* loss likely contributes to RTK inhibitor insensitivity in glioblastoma (Mellinghoff et al. 2005, 2007).

Effectively inhibiting the PI3K signaling pathway is challenging because the cascade and its feedback regulation remain incompletely understood. Specifically, RTK-activated PI3K signaling does not always require AKT, as EGFR can also signal to mTORC1 through protein kinase C (PKC) independently of AKT (Fan et al. 2009), perhaps through PDK1 (Dutil et al. 1998; Le Good et al. 1998). Indeed, a novel PI3K/PDK1/PKCι pathway can regulate phosphorylation and inactivation of the proapoptotic protein Bad, thereby increasing glioma cell survival (Desai et al. 2011). Additional work has revealed the existence of an AKT-independent, mutant PIK3CA signaling pathway leading to PDK1-mediated activation of a critical downstream effector, SGK3, in *PTEN* intact tumor cells (Vasudevan et al. 2009). PI3K pathway inhibition is thought be cytostatic, rather than cytotoxic, potentially due to G_1 cell cycle arrest (Paternot and Roger 2009; Fan et al. 2010). Combination mTOR and MEK inhibition has therefore been used that suppresses CDK4 phosphorylation in a synergistic manner (Paternot and Roger 2009). Due to complex feedback pathways, other combination

therapies being explored to target PI3K signaling in glioma include inhibition of PI3K and mTOR with inhibitors such as PI-103 (Fan et al. 2006), as well as simultaneous inhibition of mTORC1 and mTORC2 (Q Liu et al. 2011). Monotherapy with the mTORC1 inhibitor rapacmycin disrupts an IRS-1-mediated negative feedback loop and can actually increase AKT activity (Fan et al. 2006; Cloughesy et al. 2008) through mTORC2-mediated phosphorylation (Gulati et al. 2009). Interestingly, dual PI3K/mTOR inhibition with PI-103 induces autophagy, which, when also inhibited pharmacologically, leads to apoptosis (Fan et al. 2010). Thus, a combinatorial therapeutic strategy for targeting the PI3K pathway in glioblastoma will likely be necessary as more of its complex biology and regulation are revealed (Akhavan et al. 2010).

PTEN PTEN directly antagonizes PI3K signaling and is one of the most frequently altered genes in cancer. It undergoes genomic loss, mutation, or epigenetic inactivation in 40%–50% of gliomas, resulting in high levels of PI3K activity and downstream signaling (Koul 2008). As the PI3K pathway is a driving force in gliomas, even small changes in the expression or function of its critical negative regulator, PTEN, have profound effects on tumor cell behavior. The stability of PTEN is regulated post-translationally by GSK3-mediated phosphorylation at Thr 366 (Maccario et al. 2007) and by proteasomal degradation through polyubiquitination by the HECT domain ubiquitin ligase NEDD4-1 (Wang et al. 2007). NEDD4-1 up-regulation has been recently associated with overexpression of the FoxM1B transcription factor in gliomas (Dai et al. 2007). Moreover, a multiprotein tumor suppressor network exists in glioblastoma comprised of PTEN, the adaptor protein Na^+/H^+ exchanger regulatory factor (NHERF1), and the pleckstrin homology domain leucine-rich repeat protein phosphatase 1, which form a heterotrimeric complex that undergoes disruption in high-grade tumors (Molina et al. 2012). Thus, in gliomas where *PTEN* is not deleted, mutated, or epigenetically silenced, mechanisms such as aberrant up-regulation of NEDD4-1 or loss of NHERF1 could contribute to suppressing PTEN function. These mechanisms raise the possibility that the level of PTEN dysfunction is underestimated solely on the basis of genomic data.

The physiologic relevance of *PTEN* loss was underscored using several genetically engineered mouse models. CNS-specific *GFAP-Cre-p53*$^{lox/lox}$*PTEN*$^{lox/+}$ mice developed malignant gliomas with short latency that was strikingly similar to human disease both histopathologically and molecularly, given the degree of concomitant RTK activation (Zheng et al. 2008). Moreover, dual loss of p53 and PTEN in this model promoted increased c-Myc activity, which led to impaired differentiation and stable tumorigenic capacity of glioma tumor-initiating cells. Mice conditionally lacking p53, PTEN, and Rb also formed glioblastoma with SCNAs recapitulating those seen in human tumors (Chow et al. 2011). Furthermore, successive loss of each *PTEN* allele in the *NF1*$^{-/-}$ *p53*$^{-/-}$ model of progressive astrocytoma accelerated formation of grade III astrocytomas and progression into glioblastoma (Kwon et al. 2008). Together, these models demonstrate the causal role of *PTEN* loss in glioblastoma and provide the means with which to study patient-relevant disease in tractable murine models.

Clinically, *PTEN* loss has been shown to confer resistance to EGFR inhibitors in patients harboring EGFRvIII-expressing glioblastoma in part due to its activation of downstream AKT (Mellinghoff et al. 2005) as well as loss of its RTK degradation function (Vivanco et al. 2010). PTEN has also been implicated in the ubiquitin-mediated control of protein stability with respect to apoptosis, as it influences the half-life of the anti-apoptotic protein $FLIP_S$ (Panner et al. 2009). With respect to development, *PTEN* loss may also promote a "side population" phenotype of glioma stem-like cells by driving expression of the drug transporter protein ABCG2 (Bleau et al. 2009). Furthermore, recent studies have described new roles for PTEN in metabolism and cellular homeostasis; in *PTEN*-null cells, the enzyme ectonucleoside triphosphate diphosphohydrolase 5 (ENTPD5) promotes protein *N*-glycosylation and folding in the setting of increased AKT-mediated anabolism, increases growth factor receptor levels, contributes to increased aerobic glycolysis (i.e., the Warburg effect), and is required for *PTEN*-null tumor cell growth (Fang et al. 2010), although this result awaits validation in glioma models. The nuclear localization of PTEN may also play an important role in tumor suppressor activity (Perren et al. 2000; Whiteman et al. 2002; Zhou et al. 2002). Specifically, nuclear PTEN interacts with APC/C to promote formation and enhance the tumor-suppressive ability of the APC–CDH1 complex in a phosphatase-independent manner (Song et al. 2011). This demonstrates that loss and mutation of *PTEN* are not synonymous, as cells in these respective states are differentially sensitive to pharmacological inhibition of APC–CDH1 targets. Further work is necessary to fully understand which of the many functions attributed to PTEN play key roles in human glioblastomas.

NF1 Neurofibromin, the product of the *NF1* gene, is a potent tumor suppressor that negatively regulates Ras and mTOR signaling in astrocytes. Inactivation of NF1 has been observed in gliomas and can arise as a result of excessive proteasomal degradation mediated by hyperactivation of PKC (The Cancer Genome Atlas Research Network 2008; McGillicuddy et al. 2009) or by genetic loss or mutation, which were surprising findings from large-scale sequencing analyses (Parsons et al. 2008). *NF1* mutations are most commonly found in the mesenchymal subtype of glioblastoma (Verhaak et al. 2010). Experiments using *NF1*-deficient primary murine astrocytes have revealed that NF1 loss results in increased cell proliferation and migration that is dependent on Ras-mediated hyperactivation of mTOR. In this setting, mTOR induces rapamycin-sensitive activation of Rac1 GTPase that is independent of elongation factor 4E-binding protein 1(4EBP-1)/S6 kinase (S6K) (Sandsmark et al. 2007). In addition, *NF1* deficiency causes hyperproliferation and impacts glioma formation in a manner

that is independent of the tuberous sclerosis complex (TSC)/Ras homolog enriched in brain (Rheb) control of mTOR (Banerjee et al. 2011). Other potential downstream targets of neurofibromin include Stat3, which was identified in a chemical library screen using *NF1*-deficient malignant peripheral nerve sheath tumor cells (Banerjee et al. 2010). In this case, Stat3 is regulated in an mTORC1 and Rac1-dependent manner and increases cyclinD1 expression. Additionally, it will be important to determine whether *NF1*-deficient gliomas are as susceptible to proteoxicity through HSP0/mTOR inhibition as in other NF1-deficient cancers (De Raedt et al. 2011).

Genetically engineered mouse models have demonstrated that targeted homozygous loss of *NF1* in astrocytes, while sufficient to increase cell growth in vitro and in vivo, is not sufficient to induce glioma formation (Bajenaru et al. 2002). Interestingly, $NF1^{-/-}$ astrocytes develop optic gliomas in the context of an $NF1^{+/-}$ brain environment (Bajenaru et al. 2003; Zhu et al. 2005), in part through paracrine factors including hyaluronidase (Daginakatte and Gutmann 2007). In addition, it has been found that low levels of cAMP expression in the stroma are sufficient to induce optic glioma formation in genetically engineered mouse models of NF1 such that local depletion of cAMP resulted in glioma formation in regions of the brain otherwise not observed to develop tumors (Warrington et al. 2010). These findings emphasize the importance of heterogeneity and the cell type-specific effects of various genetic alterations in tumorigenesis. Other genetically engineered mouse models have demonstrated that *NF1* loss in glial cells, in combination with a germline *p53* mutation, results in fully penetrant malignant astrocytomas (Zhu et al. 2005), which progress to glioblastoma upon deletion of *PTEN* (Kwon et al. 2008). More recent work has revealed that the same combination of genetic alterations in these tumor suppressor genes in neural stem/progenitor cells is necessary and sufficient to induce astrocytoma formation (Alcantara Llaguno et al. 2009).

Mutant IDH1/2 proteins

Mechanistic basis of mutant IDH1 biology Mutant IDH1/2 have been shown to catalyze a neomorphic function (Dang et al. 2010). Specifically, while wild-type IDH1 catalyzes the $NADP^+$-dependent oxidation of isocitrate, mutant IDH1 catalyzes the NADPH-dependent reduction of α-KG to the (R)-enantiomer of 2-hydroxyglutarate (2-HG), which is the same stereoisomer of 2-HG that is seen in D-2-HGA. Although wild-type IDH1 can also catalyze this particular reaction (Pietrak et al. 2011), mutant enzymes perform this reaction with much higher efficiency because the R132 substitutions modify the active site to increase α-KG and NADPH binding (Dang et al. 2010; Pietrak et al. 2011). Dang et al. (2010) showed that mutant cells contained extremely high levels of 2-HG, which was also found in primary *IDH1* mutant gliomas and in the serum of *IDH* mutant AML patients (Gross et al. 2010; Ward et al. 2010).

Work on the downstream biological effects of *IDH1/2* mutation expression has focused largely on the inhibition of α-KG-dependent dioxygenases by 2-HG. This diverse group of enzymes controls a broad range of physiological processes, including hypoxic sensing, histone demethylation, demethylation of hypermethylated DNA, fatty acid metabolism, and collagen modification, among others (Loenarz and Schofield 2008). Several studies have provided evidence to demonstrate that several of these functions are influenced by IDH1/2 mutation expression. *IDH1* mutant gliomas exhibit a global DNA hypermethylation state, termed the glioma CpG island methylator phenotype (G-CIMP) (Noushmehr et al. 2010). This state was also observed in *IDH1/2* mutant AML (Figueroa et al. 2010). Mutant IDH1 expression was sufficient to produce a G-CIMP phenotype in engineered normal human astrocytes that was highly concordant with that seen in *IDH* mutant human tumor samples. Furthermore, this methylation phenotype correlated with a gene expression signature comprised of a limited set of down-regulated genes that discriminated between *IDH1* mutant and wild-type proneural tumors. This hypermethylation state may be caused in part by the 2-HG-mediated inhibition of the α-KG-dependent TET2 enzyme (Xu et al. 2011; Turcan et al. 2012); the resultant decrease in 5-hydroxymethylcytosine was also observed in glioblastoma specimens (Xu et al. 2011). Moreover, mutant *IDH1/2* cells displayed impaired hematopoietic differentiation, suggesting that a hypermethylated epigenetic landscape contributed to a persistent dedifferentiated state (Figueroa et al. 2010). The inhibition of histone demethylases in *IDH1* mutant cells may also impair differentiation (Chowdhury et al. 2011; Xu et al. 2011; Lu et al. 2012). Repressive histone methylation was shown to be associated with impaired mutant IDH1-expressing astrocyte differentiation, and the accumulation of these histone marks preceded significant DNA hypermethylation in engineered *IDH1* mutant cells (Lu et al. 2012). Furthermore, Lai et al. (2011) showed that global expression profiles of *IDH1* mutant glioblastomas more closely resembled lineage-committed neural precursors, whereas wild-type counterparts appear to resemble neural stem cells.

The production of 2-HG also appears to influence HIF biology in several ways. First, 2-HG may stabilize HIF-1 under some conditions (Zhao et al. 2009). However, the HIF-1 response to hypoxia in *IDH* mutant cells is attenuated (Koivunen et al. 2012). Specifically, the (R)-enantiomer of 2-HG stimulates the EGLN prolyl 4-hydroxylases, which mark HIF for degradation. Either expression of mutant IDH1, suppression of HIF-1α, or overexpression of EGLN1 was sufficient to stimulate colony formation by immortalized human astrocytes, and HIF-regulated genes were down-regulated in *IDH1* mutant proneural tumors. Hypoxic cells drive lipogenesis via reduction of glutamine to α-KG by wild-type IDH1 (Metallo et al. 2012). This observation suggests a mechanism by which cells can survive under hypoxic or pseudohypoxic conditions and points to a physiological selection pressure to maintain a copy of the wild-type *IDH1* gene in cancer cells. Finally, mutant IDH1/2 expression or

2-HG administration can potently inhibit multiple histone demethylases, likely altering transcriptional programs. Additional work has shown that cells expressing the IDH1 R132H mutation display metabolomics alterations in amino-free and branched chain amino acid levels and also choline phospholipid synthesis (Reitman et al. 2011). Thus, together, these data have pointed to several biological processes affected by mutant IDH1/2 expression that, collectively, may promote tumor growth by integrating changes in development, global transcriptional programs, metabolism, and responses to hypoxia.

Several studies suggest that *IDH1/2* mutation may be an early event in *IDH1/2* mutant neoplasms. When patients with diffuse astrocytoma or oligoastrocytoma were subjected to serial biopsies, there were seven patients who carried only *IDH1* mutations at the first biopsy but acquired either *TP53* mutation or 1p19q loss at the second biopsy, suggesting a temporal sequence of mutation acquisition. This possibility is also supported by recent sequence analysis of *IDH1* and *p53* genes in a separate study by Lai et al. (2011). Analysis of a large panel of grade II–IV astrocytomas showed a higher propensity for Arg-to-Cys substitutions at position 273 in *p53* compared with the high rate of Arg-to-His substitutions at position 132 in *IDH1*, which would be consistent with a strand asymmetry mechanism (Rodin and Rodin 1998) in which C → T mutations took place on the transcribed strand in *IDH1* but on the nontranscribed strand in *p53*. The result of this mutational asymmetry would be that mutant IDH1 could be expressed in a nonreplicating clone, whereas mutant p53 could be expressed only after DNA replication in S phase. Together, these studies provide potential insights into the evolution of *IDH1* mutant cancers and highlight the importance of serial tissue analysis and the need for careful clonal analysis to fully clarify how these cancers progress.

Translational relevance of IDH1 *status* Despite our incomplete understanding of mutant IDH biology, the mutant status of the *IDH1/2* genes may serve as an important prognostic indicator. Specifically, patients with anaplastic astrocytoma (Parsons et al. 2008; Sanson et al. 2009; Yan et al. 2009; Hartmann et al. 2010) and glioblastoma (Yan et al. 2009) harboring mutant IDH1 demonstrate a significantly longer overall survival compared with wild-type IDH1 counterparts and are younger at presentation, and this survival benefit has also been observed in grade II gliomas (Sanson et al. 2009). Patients with G-CIMP$^+$ tumors also experience a similar survival benefit (Noushmehr et al. 2010). In addition, a comprehensive genomic and clinical analysis of glioblastomas harboring mutant and wild-type *IDH1* suggests that, while histopathologically similar, these tumors may represent disease processes far more disparate than has been appreciated. Specifically, *IDH1* mutant tumors display less contrast enhancement, less peritumoral edema, larger initial size, greater cystic components, and a greater likelihood of frontal lobe involvement compared with wild-type tumors (Lai et al. 2011). In addition, several methods have been developed to assess IDH1 mutant protein status (Capper et al. 2009) or its 2-HG by-product (Sahm et al. 2012) in clinical settings. While 2-HG is easily detected in the serum of AML patients, the correlation between serum 2-HG and tumor mutation status may be less specific in the setting of glioma (Capper et al. 2011). Finally, it may also be possible to monitor the presence of 2-HG noninvasively using magnetic resonance spectroscopy (MRS) imaging of the brain (Pope et al. 2012). Together, these studies provide overwhelming evidence for the clinical relevance of *IDH1* status in gliomas from grades II to IV and support the proposal that its status be incorporated into the current WHO histopathological scheme for every glioma analyzed (Hartmann et al. 2010). Despite their histological similarities, *IDH1* mutant and wild-type glioblastomas are clearly distinct diseases (Fig. 1), and understanding the biological basis behind the differences in their natural histories will surely be a major area of focus in the field.

Tumor biological hallmarks in glioma: invasion, angiogenesis, and tumor-initiating cells

Glioma cell invasion

The ability of glioblastoma cells to invade adjacent brain tissue contributes to the major clinical problem in achieving disease control. The majority of glioblastomas treated initially with standard therapy will recur within several centimeters of the initial tumor location (Hochberg and Pruitt 1980; Chamberlain 2011). In addition, 10%–20% of patients may harbor "macroscopic" evidence of invasion at the time of presentation, including multifocal disease—which can even be bihemispheric in the so-called "butterfly" pattern—at noncontiguous sites in the brain or involving spread along white matter tracts and through subependymal and/or subarachnoid spaces (Parsa et al. 2005; Chamberlain 2011). It is well established that histologically identifiable tumors invariably greatly exceed the area of disease detected radiographically (Burger et al. 1988). The intrinsic capacity of glioblastoma to invade was well illustrated by classic autopsy studies, which demonstrated that 25%–50% of untreated glioma patients examined harbored histological evidence of bilateral disease (Scherer 1940; Matsukado et al. 1961). Notably, even grade II, or "diffuse," gliomas exhibit invasive capacity such that cells derived from this tumor grade can be observed histologically at least 2 cm from the main tumor mass when "supratotal" surgical resections have been attempted (Yordanova et al. 2011). Thus, glioma cell invasiveness is a clinical problem in the majority of diffuse gliomas.

The process of glioblastoma invasion likely involves sequential adhesion to the extracellular matrix (ECM), degradation of the ECM, and altered cell contractility (Giese and Westphal 1996; Nakada et al. 2007; Tate and Aghi 2009; Onishi et al. 2011). Unlike non-CNS cancers, clinically manifest hematogenous or lymphatic spread of glioblastoma outside the brain is exceedingly rare (Hoffman and Duffner 1985). Also, the ECM composition and distribution in the brain is distinct from other extra-CNS tissue sites. A typical collagen-rich basement membrane

ECM exists in the glia externa limitans, which covers the cortical surface, and also surrounding cerebral blood vessels (Rutka et al. 1988; Louis 2006; Gritsenko et al. 2012). However, the brain parenchyma harbors a unique ECM structure, the perineuronal network, which is a meshwork composed predominantly of hyaluronan sulfate proteoglycans as well as chondroitin sulfates proteoglycans, tenascins, and link proteins (Kwok et al. 2011; Gritsenko et al. 2012). Moreover, distinct brain regions, such as the neurogenic areas of the SVZs, are more enriched for chondroitin and heparan sulfate proteoglycans (Sirko et al. 2007).

Glioma cells adhere to the ECM using several mechanisms. The immunoglobulin superfamily member CD44 and the hyaluronan-mediated motility receptor (RHAMM), both receptors for hyaluronan, are expressed in glioblastoma (Akiyama et al. 2001). CD44 is cleaved by both ADAM proteases (Murai et al. 2004) and MMP-9 (Chetty et al. 2012) in a process that promotes motility via cytoskeletal reorganization (Murai et al. 2004; Bourguignon 2008); it is likely that myosin II is also important for glioma cell contractility during invasion (Beadle et al. 2008). Strikingly, CD44 and RHAMM are both suppressed by p53 (Godar et al. 2008; Sohr and Engeland 2008), suggesting that early cellular progression through canonical checkpoints and the ability to migrate/invade are linked, although this possibility remains to be validated in glioma models. Integrins, particularly the αvβ3 and αvβ5 heterodimers (Bello et al. 2001a), also likely contribute to glioma cell adherence to the ECM in a process that activates cytoskeletal rearrangement through cytoplasmic mediators, including FAK (Rutka et al. 1999; Riemenschneider et al. 2005) and/or Pyk2 (Lipinski et al. 2008). Their putative involvement in glioma pathobiology (D'Abaco and Kaye 2007; Desgrosellier and Cheresh 2010) has prompted the testing of the αvβ3 and αvβ5 integrin inhibitor cilengitide in an ongoing phase III CENTRIC clinical trial for newly diagnosed glioblastoma in combination with radiation therapy and temozolomide (Reardon et al. 2011a).

Several MMPs have been implicated in modifying the ECM in the local microenvironment to promote invasion (Rao 2003). Recent work revealed a novel mechanism of EGFR-mediated invasion in which EGFR-dependent up-regulation induced high expression of the IFN-regulated factor guanylate-binding protein 1, leading to increased glioma invasion through MMP-1 expression (Li et al. 2011). MMP-2 and MMP-9 also drive glioma invasion (Forsyth et al. 1999) and are regulated through several molecular cascades. Both enzymes promoted invasion when up-regulated via a CD95-mediated activation of AKT1 involving recruitment of Src family member Yes and p85. Importantly, CD95L expression was demonstrated at the leading glioma edge in clinical samples (Kleber et al. 2008). MMP-2 and MMP-9 were also coordinately up-regulated by the low-density lipoprotein receptor-related protein 1 in an ERK-dependent promigratory process (Song et al. 2009). Further work has revealed additional regulators of MMP-2 expression, including the PTEN-regulated insulin growth factor-binding protein 2 (IGFBP2) (Wang et al. 2003; Levitt et al. 2005) and the forkhead transcription factor FoxM1B. FoxM1B not only up-regulated MMP-2 and was overexpressed in human glioblastoma specimens (Dai et al. 2007), but also transformed engineered, immortalized normal human astrocyte cells into invasive glioblastoma cells via a pathway involving PTEN degradation and AKT activation (Dai et al. 2010). Notably, AKT also regulates the actin-binding protein Girdin, which directs neural cell migration and contributes to tumor-initiating cell invasiveness (Natsume et al. 2011); because several invasive mechanisms can be regulated by the PTEN/PI3K/AKT pathway, its contribution to this phenotype deserves further investigation. Given that the MMP inhibitor marimistat did not show efficacy in a randomized clinical trial, albeit as a monotherapy without temozolomide (Levin et al. 2006), it will be important to further clarify both the critical proteases and their regulatory pathways that promote glioblastoma invasiveness.

A growing number of additional molecules have been implicated in invasion and are reviewed elsewhere (Nakada et al. 2007; Teodorczyk and Martin-Villalba 2010). Importantly, it will be critical to test candidate proinvasive targets and programs in physiologically relevant tumor models with aggressive growth behavior. Conventional malignant glioma cell lines do not tend to exhibit invasive growth in orthotopic xenograft models, but tumor-initiating cells not only invade (Singh et al. 2004; Wong et al. 2011), but also often phenocopy the type of invasion observed radiographically in patients from which the cells were derived (Wakimoto et al. 2012), thereby representing high-fidelity platforms to explore invasion (Bhat et al. 2011). Furthermore, the striking, but poorly understood, migratory capacity of neural and mesenchymal stem cells in transplant models (Carney and Shah 2011) affords another invasive model that can be used to interrogate this process.

Angiogenesis

Angiogenesis plays an important role in glioblastoma (Kargiotis et al. 2006), as evidenced by the presence of microvascular proliferation (Louis et al. 2007). The recent demonstration that therapeutic strategies to inhibit components that contribute to angiogenesis have been shown to have some efficacy in glioblastoma (Reardon et al. 2011b), including the recent approval of bevacizumab, provides the foundation for future studies in this disease.

Glioma cells require blood vessels for metabolic purposes, such as oxygen and nutrient delivery and waste removal, and also for the creation of a vascular niche that may selectively support glioma stem cells (Calabrese et al. 2007; Gilbertson and Rich 2007). The development of glioma vasculature may occur through several mechanisms (Carmeliet and Jain 2011a): angiogenesis, the formation of new blood vessels from the existing vasculature (Folkman 1971; Kerbel 2008); vasculogenesis, which involves the recruitment of bone marrow-derived endothelial progenitor cells (Patenaude et al. 2010); re-

cruitment of tumor cells directly into the vascular wall; or the differentiation of tumor stem cells directly into vascular endothelium (Ricci-Vitiani et al. 2010; Wang et al. 2010). The end result of these blood vessel-producing processes is an intratumoral vasculature that is highly aberrant, incomplete, and tortuous (Long 1970), creating areas of hypoxia, acidosis, and peritumoral edema.

Blood vessel formation is regulated by a balance between pro- and anti-angiogenic molecules that comprise an angiogenic switch (Bergers and Benjamin 2003). VEGF, acting through VEGFR-2/KDR, is believed to be the central proangiogenic factor and is induced by hypoxia via HIF-1α (Shweiki et al. 1992; Kaur et al. 2005) and several mitogenic pathways that are dysregulated in glioblastoma (Maity et al. 2000; Pore et al. 2003; Watnick et al. 2003; Phung et al. 2006). VEGFR-2 activation then regulates endothelial cell survival, proliferation, migration, and permeability (Hicklin and Ellis 2005). In addition to VEGF, there are a large number of other factors that stimulate angiogenesis in glioblastoma (Carmeliet and Jain 2011b), including PDGF, FGF, the ANG/TIE system, Notch signaling, integrins, ephrins, IL-8, and SDF-1α (Carmeliet and Jain 2011a; Weis and Cheresh 2011). These proangiogenic mediators are opposed by anti-angiogenic factors, including angiostatin, thrombospondins, endostatin, tumstatin, and interferons (Nyberg et al. 2005). When stimulatory factors outweigh inhibitory factors, the angiogenic switch favors blood vessel creation.

Several types of angiogenesis inhibitors have been developed for therapeutic use. Because it is the main driver of angiogenesis, most approaches target VEGF signaling by interfering with either the VEGF ligand (Vredenburgh et al. 2007b), its receptor (VEGFR-2/KDR) (Batchelor et al. 2010), or its downstream signaling cascade. The anti-VEGF antibody bevacizumab is now used heavily in the clinic after undergoing accelerated approval by the FDA for use in recurrent glioblastoma (Cohen et al. 2009). Two initial phase II trials of bevacizumab and irinotecan demonstrated a 60% radiographic response and an apparent doubling of 6-mo progression-free survival (PFS; 38%–46%) and median survival (40–42 wk) compared with historical controls (9%–15% and 22–26 wk, respectively) (Vredenburgh et al. 2007a,b). In addition, two follow-up phase II trials demonstrated a radiographic response (27%–38%), increased PFS (29%–50%), and a slightly prolonged overall survival (31–35 wk) compared with historic controls (Friedman et al. 2009; Kreisl et al. 2009); phase III trials, including the RTOG-0825 study, are currently under way (http://www.clinicaltrials.gov). Additional approaches, such as angiopoietin inhibitors, are reviewed elsewhere (Norden et al. 2008a; Reardon et al. 2011b).

However, several unanswered clinical questions remain that require an improved understanding of the basic mechanisms involved in glioma angiogenesis (Verhoeff et al. 2009). For example, anti-angiogenic agents reduce vasogenic edema and corticosteroid requirements by decreasing vascular permeability (Gerstner et al. 2009), thereby dramatically modifying the MRI appearance of glioblastoma (Wen et al. 2010). However, it is unclear whether these changes result from "vascular normalization" (Jain 2005; Carmeliet and Jain 2011b), actions on tumor cells, or other alterations in the blood–brain barrier (Bechmann et al. 2007). In addition, patients on these agents tend to progress rapidly when disease recurs after treatment with anti-angiogenic agents (Ellis and Hicklin 2008), with minimal response to subsequent chemotherapy (Quant et al. 2009), underscoring the importance of understanding the basis for treatment resistance. Due to compensatory up-regulation of alternative angiogenic (Batchelor et al. 2007; Sathornsumetee and Rich 2007) or vasculogenic (Du et al. 2008) pathways, combinations of inhibitors that target nonredundant vascular pathways may be necessary. In addition, there is some evidence that angiogenesis inhibitors may promote infiltrative glioma growth (Norden et al. 2008b; Iwamoto et al. 2009; Narayana et al. 2012). An improved understanding of the basic biology of angiogenesis, the mechanisms of resistance to inhibitors, and the contributions of tumor-initiating cell-derived vasculature to the microenvironment will be necessary to optimize angiogenesis inhibition as a therapeutic approach in glioblastoma.

Glioblastoma ontogeny: cellular origins and tumor-initiating cells

Cellular origins The cellular origins of malignant gliomas continue to be a source of debate. As in other cancers, the continued interest in glioma ontogeny is stimulated by the possibility that an improved understanding of the normal cell of origin will help identify fundamental pathways and lineage dependencies that could represent novel diagnostic and therapeutic targets (Visvader 2011). Specifically, numerous studies in genetically engineered mouse models have provided evidence that gliomas arise from the normal reservoirs of cycling stem and progenitor cells within the brain, and numerous genetically engineered mouse models have supported this idea in that a diverse range of glioma-relevant mutations targeted to neural stem cells in vivo readily produce gliomas with high fidelity and penetrance (Bachoo et al. 2002; Zhu et al. 2005; Zheng et al. 2008). For instance, tamoxifen-inducible *Cre*-recombinase-mediated inactivation of *p53* and *NF1* in adult stem cells drove the consistent formation of astrocytomas (Alcantara Llaguno et al. 2009; Wang et al. 2009); interestingly, these tumors were not restricted to the SVZ neural stem cell niche location but were found within multiple other brain regions. This finding was extended by C Liu et al. (2011) using the mosaic analysis with double markers (MADM) technique (Zong et al. 2005). This approach enabled careful longitudinal, lineage tracing analysis of developing astrocytomas in mice with mosaic *p53/NF1* mutant neural stem cells, which showed that the accelerated phase of tumor growth occurs not in the original cell of mutation, but within $OLIG2^+$ oligodendroglial progenitor cells (OPCs) that migrate away from the niche zones (C Liu et al. 2011). The finding that "astrocytomas" can arise from OPCs in mice could help to explain the long-standing observation that human

glioblastoma and all other astrocytomas are characterized by both expression of oligodendroglial markers to an even greater degree than astrocytic markers (Ligon et al. 2004; Phillips et al. 2006; Verhaak et al. 2010) and their dependency on the same lineage factors (e.g., OLIG2) as normal counterparts (Ligon et al. 2007). Distributed progenitor cells, such as OPCs, actually represent the largest pool of cycling cells in the brain and are defined by expression of OLIG2 and NG2 (Dawson et al. 2003; Geha et al. 2010). Targeting of these nonstem cell progenitors through transgenic or viral approaches has been shown to lead to malignant astrocytomas or oligodendrogliomas, depending on the combination of mutations and cell types targeted (Geha et al. 2010; Persson et al. 2010).

In addition, work in genetically engineered mice has demonstrated that gliomas may also arise from terminally differentiated cells, likely through a process of dedifferentiation. Bachoo et al. (2002) demonstrated that cultured mature *Ink4a/Arf*$^{-}$ astrocytes stimulated with EGF or expressing mutant EGFRvIII dedifferentiated to nestin-expressing progenitor cells capable of forming high-grade gliomas in vivo, providing evidence that both progenitor and lineage-restricted, mature cells were permissive contexts for glioma formation. Moreover, PDGF has also been shown to induce the dedifferentiation of mature astrocytes and induce glioma formation in GFAP-expressing *Ink4a/Arf*$^{-}$ cells in vivo (Dai et al. 2001). Together, these studies emphasize the influence of particular combinations of dysregulated pathways on glioma development and highlight the notion that specific genetic alterations, and not just the precise a priori developmental state of the cell, help to create permissive contexts for glioma formation.

Although murine studies have been helpful in clarifying glioma origins, it remains to be seen whether their findings can be fully extrapolated to human disease. For instance, human and mouse OPCs may be biologically similar, as recent work demonstrated conserved mechanisms for oligodendroglioma formation through disruption of asymmetric division of NG2^{+} OPCs in mouse *verbB/p53*$^{+/-}$-induced oligodendrogliomas and human oligodendrogliomas (Sugiarto et al. 2011). However, in adult mice, the SVZ is a prominent regenerative zone, but the analogous region in adult human brains, the subventricular astrocyte ribbon, may not harbor similar numbers or types of stem cells (Sanai et al. 2011). In addition, the plasticity of cells in response to engineered oncogene and tumor suppressor alterations is well established and thus can confound deconvolution of tumor-initiating populations from established tumors. For instance, studies of the effects of Smo activation on development of a mouse model of medulloblastoma suggested that the same mutation at multiple stages of lineage commitment could result in dedifferentiation of committed cells to a common phenotypic end point, thereby obscuring the original origin of the tumors (Schuller et al. 2008). It will be important to continue to model human disease in mice given the powerful technologies enabling sophisticated genetic targeting. These approaches will be invaluable to explore clinical observations such as the distinct differences in location between *IDH1* mutant and wild-type glioblastomas (Lai et al. 2011). Given the diverse number of histological subtypes, various subsets of molecular patterns and subclasses, and increasingly broad number of stem and progenitor cells in the brain, further studies will be required to clarify the origins of malignant gliomas and the programs that drive their subsequent evolution.

Tumor-initiating cells Complementing these studies of the cell of origin in glioblastoma, several groups have shown that different cell populations within a tumor exhibit varied tumor-forming capacity. These observations suggest that stem/progenitor cells may serve as the cell of origin for many tumors. Irrespective of the ontogeny argument, however, the finding that distinct cellular subpopulations within glioblastomas harbor potent tumor-initiating capacity was yet another example that heterogeneity—morphological, molecular, and cellular—is the rule, rather than the exception, in this group of diseases. First described by several groups (Ignatova et al. 2002; Hemmati et al. 2003; Singh et al. 2003), the finding that tumor-initiating cancer cells can be isolated from glioblastomas and other cancers and propagated as neurospheres when grown in serum-free neural stem cell conditions, have self-renewal capacity, and recapitulate malignant glioma behavior in vivo (Singh et al. 2004) has transformed glioblastoma research. Moreover, these cells in malignant gliomas now represent well-characterized experimental models for the investigation of the cancer stem cell hypothesis (Dirks 2010), since these cells represent highly faithful glioma models when cultured as tumor-initiating cell lines or orthotopic xenografts. For instance, culturing tumor specimens in stem cell conditions has enabled the maintenance of lines carrying glioma-specific lesions such as EGFRvIII (Stockhausen et al. 2011) or mutant IDH1 expression (Luchman et al. 2012) that were not well-modeled under serum-containing conditions. In addition, abundant evidence confirms that these models recapitulate the phenotypes as well as genomic alterations found in patient tumors (Lee et al. 2006a; Wakimoto et al. 2012). The glioma stem cell hypothesis (Dirks 2010; Taylor et al. 2010; Lathia et al. 2011a) incorporates a model in which these tumor-initiating cells are the principal drivers of gliomagenesis. Specifically, these cells are thought to represent transformed neural progenitors that give rise to tumor cell progeny and also have the capacity to self-renew. Considering the high malignant potential of the tumor-initiating cell population, this hierarchical model implies that this subset, which may represent a minority of cells within the bulk tumor mass, is the critical subpopulation of glioblastoma that could be highly relevant clinically due to the potential for intrinsic therapeutic resistance (Bao et al. 2006). In contrast, an alternative view of gliomagenesis involves a less hierarchical model in which most glioblastoma cells are functionally and developmentally similar and have developed through a progressive process of clonal selection of transformed somatic cells. In this case, there may be a higher proportion of the tumor mass with

malignant potential than in the stem cell model. Further work will be important to discern the physiological relevance and potential translational impact of these not mutually exclusive models.

Although the tumor-initiating cell subpopulation has been traditionally defined by the cell surface expression of CD133, it has become clear that this marker does not exclusively define tumor-initiating populations (Ogden et al. 2008; Chen et al. 2010; Beier and Beier 2011), and thus further work correlating cell surface markers (Lathia et al. 2011b) with phenotype is necessary to more clearly define these cells. Moreover, while substantial work has begun to clarify the programs—including PLAGL2, Olig2, and c-Myc (Ligon et al. 2007; Zheng et al. 2008, 2010; Guryanova et al. 2011)—that drive and maintain tumor-initiating characteristics, additional study is needed regarding the molecular pathways underlying cellular phenotype and hierarchies. Also, the degree to which these cells strictly preserve the phenotypes of all genotypes of original patient tumors remains to be examined in large numbers of patients in detail; reports of variation in respect to preservation of RTK, bias toward tumors with PTEN inactivation, and difficulties in modeling the vascular changes seen in human glioblastoma suggest that careful attention to the unique properties of each line will be required (Chen et al. 2010). Nevertheless, the use of glioblastoma tumor-initiating cell models in the basic research setting is now routine, and numerous emerging preclinical studies using patient-derived models will provide clues as to whether these lines gathered into patient cohorts might advance drug screening efforts (Pollard et al. 2009).

Future directions: areas of further study

Identifying dependencies and targets

Despite the tremendous progress in our understanding of the genetic basis of glioblastoma, targeted therapeutic approaches based on known genomic alterations have not proved to be efficacious to date (De Witt Hamer 2010). It is likely that intratumoral heterogeneity and target cooperativity (Stommel et al. 2007) conspire to create a "multiple dependency" state wherein single-target inhibitors are not sufficient to attenuate tumor growth. Moreover, transcriptionally defined subclasses may harbor unique dependencies. In addition, we have an incomplete understanding of the functional consequences of many of the genes found mutated in glioblastoma. Indeed, it will be necessary to approach functional space in glioblastoma in the systematic manner with which the structural space has been clarified (Boehm and Hahn 2011). To this end, genome-wide tools capable of loss- and gain-of-function studies will likely be invaluable in interrogating phenotypes particularly relevant to glioblastoma. Large-scale pooled shRNA screening of a panel of glioblastoma cell lines as part of a larger panel of multiple lineages led to the identification of a list of candidate dependencies (Cheung et al. 2011). These approaches should use panels of highly faithful models—murine and human—that recapitulate the salient in vivo behaviors of interest. In addition to survival, phenotypes such as invasion should also be assessed. Systematic approaches may also be useful in revealing synthetic-lethal relationships (Barbie et al. 2009), such as the role of ENTPD5 in the context of *PTEN* loss (Fang et al. 2010). In addition to systematic approaches, the computational methodologies involving gene expression data that have pointed to important biological roles for STAT3 (Carro et al. 2010; Bhat et al. 2011) and the Hippo pathways, among others, have shown great promise and should be explored further in preclinical contexts.

Understanding malignant transformation

The phenomenon of transformation from grade II glioma to malignant disease is poorly understood yet accounts for the mortality of low-grade disease. These patients are younger at diagnosis, and it is thought that 45%–70% will undergo disease progression to histological malignancy within 5 years (Ohgaki and Kleihues 2005). Although the entity of secondary glioblastoma represents <10% of all diagnosed glioblastomas (Ohgaki and Kleihues 2009), the existence of histologically proven antecedent lower-grade tissue presents a unique and as yet relatively underexploited opportunity to understand the programs that drive malignancy and also to explore the possibility of delaying clinical transformation. The study of cancers that progress from lower-grade lesions to higher-grade malignancies has provided critical insights into the genetic basis of cancer evolution. Specifically, analysis of the molecular changes along the histopathological spectrum in colorectal cancer (Vogelstein et al. 1988) pioneered comparative analyses of lower-grade lesions and their higher-grade counterparts. Together, the dissection of the chronological genomic events that drive disease progression not only provides invaluable fundamental mechanistic information on a cancer's natural history, but also may identify potential therapeutic targets that drive the transition from less aggressive to more malignant disease. Especially considering the comparatively young age of presentation in low-grade patients, the benefit to interrupting or delaying the transformation pathway would be significant. Moreover, despite the clear differences between *IDH1* mutant secondary glioblastoma and *IDH1* wild-type primary disease, the isolation of transformation programs identified by studying low-grade and matched malignant disease could be relevant to the programs, such as those driving angiogenesis, that contribute to the biology of primary disease. Earlier studies comparing primary and secondary glioblastomas (Maher et al. 2006; Tso et al. 2006), characterizing the expression of DCC in low- and high-grade tumors (Reyes-Mugica et al. 1997), and performing comparative array CGH between a small set of grade II/IV gliomas (Idbaih et al. 2008) point to the differences in these tumors.

Understanding the extent and impact of heterogeneity

The morphological heterogeneity that prompted the original description of high-grade glioma as "glioblastoma multi-

forme" (Mallory 1914; Bailey and Cushing 1926) has also extended to the molecular level. Through studies demonstrating variable intratumoral distributions of EGFRvIII-expressing cells (Inda et al. 2010) as well as mutually exclusive SCNAs within distinct populations of the same tumor (Snuderl et al. 2011; Szerlip et al. 2012), among others, we are beginning to have a better understanding of how molecular heterogeneity is manifest. While these studies illustrate examples of distinct alterations occurring in unique cells, there are also examples of concomitant kinase activation within the same cell (Stommel et al. 2007). In both cases, it is not entirely clear whether intermingled cell populations with unique genomic alterations behave as independent tumors or are interdependent with each other. Ultimately, it will be necessary to study the populations of a heterogeneous tumor mass at the single-cell level in order to understand co-occurring genetic alterations and accompanying protein activation states. Developing technology capable of assessing single-cell-scale genomes, isolated through laser capture microscopy or live cell-sorting techniques, is making these approaches feasible (Navin and Hicks 2011; Navin et al. 2011). Moreover, in addition to understanding the molecular basis of tumor cell heterogeneity, it will be critical to consider the contributions and phenotypes of the diverse nontumor cell types, such as stromal and immune/inflammatory cells (Dunn et al. 2007), that populate the glioma microenvironment.

Developmental oncobiology

Given the growing prominence of stem and progenitor cell biology in glioblastoma, further characterization of the heterogeneous cell populations found within these tumors will be critical in understanding their origins and evolution. Although it is clear that defects in developmental hierarchy play a key role in many tumors, further work is necessary to determine the molecular basis underlying the differentiation potential of the cells within each tumor. Specifically, comprehensive genomic, epigenomic, and transcriptional characterization of carefully isolated progenitor cells from tumors compared with their nonneoplastic counterparts may clarify the structural basis of particular cell states, whether there are distinct differences across the transcriptomic subtypes in glioblastoma, and how the neoplastic context influences phenotypes such as invasion and migration, resistance to therapy, and signaling dysregulation. Furthermore, these types of comparative studies may enable the development of therapeutic approaches that reduce the tumor-initiating/tumor-propagating capacities of glioma tumor-initiating cells by interrupting the cellular programs that likely contribute to their aggressiveness, such as self-renewal, maintenance of multipotentiality, and the phenotypic plasticity to dedifferentiate.

Therapeutic resistance

Given the inevitable progression of glioblastoma following standard-of-care surgery, temozolomide treatment, and radiation therapy (Stupp et al. 2005), it is likely that both intrinsic and acquired mechanisms of resistance play significant roles in glioblastoma. Currently, most patients continue to receive temozolomide as the main chemotherapeutic agent in their treatment regimen. In addition to the de novo resistance mechanism of *MGMT* hypomethlyation (Hegi et al. 2005), several groups have described additional mechanisms of treatment resistance that include loss of the mismatch repair gene *MSH6* (Cahill et al. 2007), silencing of the base excision repair enzyme alkylpurine-DNA-*N*-glycosylase (Agnihotri et al. 2012), and also overexpression of both inhibitor of apoptosis proteins (Ziegler et al. 2008) and multidrug resistance protein family members (Shervington and Lu 2008). Although perturbation of recurrent resistance mechanisms could enhance the therapeutic efficacy of this current workhorse agent, these studies point to the broader pivotal framework of carefully studying pre- and post-intervention tumor samples, as has been demonstrated in other cancers. Comparative post-treatment annotation will be important for additional agents, such as bevacizumab, and perhaps every emerging targeted agent that enters clinical trials. This approach will also facilitate our understanding of acquired resistance and/or de novo insensitivity (Bao et al. 2006) to radiation therapy, as the vast majority of post-treatment tissue has been subjected to up-front radiation treatment. We anticipate that components of the tumor microenvironment may also contribute to resistance in a tumor cell-extrinsic manner. Specifically, the blood–brain barrier, formed by intracerebral capillary tight junctions and astrocytic foot processes, impairs the passive transit of molecules between the vascular lumen and brain parenchyma (Huber et al. 2001) and is highly dysregulated in glioblastoma (Long 1970). This structure is known to present a challenge to effective transit of therapeutic molecules to the brain, and thus continued study of its biology in both the normal and tumor settings could enhance the efficacy of drug delivery to glioblastoma.

Toward a molecular classification of glioma

The comprehensive molecular characterization of gliomas is now starting to transform their classification (Table 1), which currently follows the consensus WHO histopathological criteria instrumental in standardizing pathological classification of these challenging tumors (Louis et al. 2007). However, a high percentage of gliomas, such as mixed oligoastrocytomas and lower-grade gliomas, remain difficult to categorize reproducibly due to considerable histological overlap. Genomic approaches applied to clinically characterized patient cohorts now clearly show that combined molecular and histological classification offers great opportunities to significantly improve clinical predictive power over use of histology alone, even for individual patients. A clear example is the group including oligodendrogliomas, grade II and III astrocytomas, and mixed gliomas, which are best defined as a subset of gliomas harboring shared genomic features of mutations in *IDH* genes and are G-CIMP$^+$ and *MGMT* methylated due to globally increased levels of methylation (Noushmehr et al. 2010).

Further subclassification of this group will likely use unique lesions in oligodendroglioma, such as 1p/19q whole-arm codeletion/translocation, *CIC* mutations, and 1p or 19q polysomy (Snuderl et al. 2009; Bettegowda et al. 2011; Yip et al. 2012), in the presence or absence of *TP53* mutations to enable reliable identification of a group in a manner not possible by histology alone. Likewise, *BRAF* fusions (Horbinski et al. 2010; Hawkins et al. 2011) and *V600E* mutants characterize additional astrocytoma subsets with clinical relevance (Dias-Santagata et al. 2011; Schindler et al. 2011; Tian et al. 2011). In contrast to the mutant *IDH*/G-CIMP$^+$ tumors, glioblastomas appear surprisingly distinct but significantly more complex genomically, making diagnostic subclassification schemes more challenging. Deeper sequencing efforts have yet to be fully completed and may yet reveal novel recurrent mutations that draw together what appears now to be a highly diverse group of tumors. Clearly, mutant *IDH* will be critical in discerning these diseases, and translation of expression profiling work into diagnostic tools is also under way (Colman et al. 2010). Additional diagnostic scenarios, such as distinction of pseudoprogression versus true tumor progression, will hopefully benefit from molecular pathology approaches. The rapid pace of genomic and epigenomic discovery in gliomas will likely necessitate the development of consensus mechanisms to deploy integrated molecular and histologic data to clinical practice to improve diagnosis and predictive capability and stratify response to therapy.

Conclusion

The commitment to genomic characterization of glioblastoma has fueled substantial progress in our understanding of this cancer in the last 5 years. The heterogeneous nature of this neoplasm, both within and across tumors, underscores the difficulty in developing efficacious treatments and provides a challenge both to annotate tumors to understand the disease and to stratify patients for trials and treatment. Already there are several efforts to connect the information obtained from retrospective, population-based genomic characterization to the individual patient in the form of multiplexed patient profiling on clinical samples (MacConaill et al. 2009; Sequist et al. 2011). Further work will be necessary to fully understand glioblastoma biology and will increasingly require integrated studies, including genomics, faithful animal models of disease (Lim et al. 2011; Wee et al. 2012), and the careful study of human tissue.

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