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Oncogenic EGFR Signaling Networks in Glioma

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The epidermal growth factor receptor (EGFR) is a primary contributor to glioblastoma (GBM) initiation and progression. Here, we examine how EGFR and key downstream signaling networks contribute to the hallmark characteristics of GBM such as rapid cancer cell proliferation and diffused invasion. Additionally, we discuss current therapeutic options for GBM patients and elaborate on the mechanisms through which EGFR promotes chemoresistance. We conclude by offering a perspective on how the potential of integrative systems biology may be harnessed to develop safe and effective treatment strategies for this disease.

Introduction

Grade IV astrocytomas (glioblastoma multiforme, GBM) are an aggressive class of adult cancers with hallmark characteristics that include rampant proliferation, necrosis, genetic instability, and chemoresistance (*1*). Because of these features, GBMs are difficult to treat and often have a poor prognosis, with median overall survival of 12 months and 2-year survival rates of less than 10% (*2*). Multiple histopathological and genetic studies, together with recent large-scale cancer gene sequencing efforts, have identified the epidermal growth factor receptor (EGFR) and its downstream signaling networks as commonly deregulated components in the primary subtype of GBM tumors, a subtype that arises de novo and afflicts the majority of GBM patients (*1*, *3*, *4*). The oncogenic role of EGFR has been functionally validated in both cell culture–based systems and animal models and is a critical driver of GBM tumorigenesis. Here, we summarize the signaling mechanisms whereby EGFR and its oncogenic mutants contribute to tumor cell behavior. Additionally, we elaborate on the mechanisms through which robust EGFR signaling networks adapt to changes that occur following therapeutic intervention, resulting in clinical chemoresistance. Finally, we offer a perspective on how signaling data and computational network modeling may be harnessed in an integrative fashion to obtain a more complete view of oncogenic EGFR networks in glioma biology.

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Overview of the EGFR Receptor Family

EGFR is the prototypical member of the ErbB/EGFR family, which consists of four members in mammals (EGFR, also known as ErbB1 or HER1; ErbB2, also known as HER2/neu; ErbB3, also known as HER3; and ErbB4, also known as HER4) that appear to have diverged from a common ancestral receptor (*5*, *6*). Together, these four receptors convert cues mediated by 13 different ligands into signals that control various components of the intracellular machinery (*6*). Depending on the particular ligand and the receptor to which it binds, members of the ErbB/EGFR family mediate various cellular processes, including cell division, migration, adhesion, differentiation, and apoptosis (*7*). Because so many fundamental cell processes are regulated by the EGFR family of receptors and ligands, deregulation of these components can lead to cancer and other diseases (*5*).

The EGFR family shares a general domain organization in which an extracellular ligand-binding region is linked through a hydrophobic transmembrane domain to a cytoplasmic region that contains both a tyrosine kinase domain and C-terminal tail (*8*). Upon ligand binding, EGFR undergoes receptor dimerization (*9*, *10*), tyrosine kinase activation, and trans-phosphorylation across receptor dimers on multiple tyrosine residues in the cytoplasmic tail (*5*, *8*). The extracellular region of the EGFR consists of four domains (I to IV) (*10*, *11*). Crystal structures of the ectodomains of EGFR, ErbB3, and ErbB4 revealed that EGFR exists in two distinct conformations (*9*–*13*). There is a closed, inactive conformation in which intramolecular interactions between domain II and IV tether each other to prevent domains I and III from coming together to form the ligand-binding site (*12*, *13*). This conformation is in equilibrium with an open active state of the receptor (*14*, *15*). In the open conformation, domains II and IV move away from domains I and III, resulting in not only the formation of the ligand-binding pocket, but also exposure of the dimerization loop in domain II for interaction with the identical domain of another EGFR molecule to form the homodimer (*10*, *11*). In the absence of ligand, the equilibrium shifts to favor the closed conformation. However, ligand binding stabilizes the open conformation and shifts the equilibrium, allowing for the accumulation of active homodimers and receptor signaling (*14*, *15*).

Receptor phosphorylation leads to the recruitment of multiple effector proteins through recognition and binding of Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains on the effector proteins to phosphotyrosine motifs on the receptor (*16*). The formation of this signaling complex results in the initiation of various downstream signaling cascades, including the phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription 3 (STAT3) pathways, which regulate a multitude of cellular responses. Receptor tyrosine phosphorylation also initiates negative regulatory mechanisms through the recruitment of ubiquitin ligases such as Casitas B-lineage lymphoma proto-oncogene (Cbl) that lead to receptor internalization and degradation (*17*).

Mechanisms of EGFR Deregulation in Glioblastoma

Receptor overexpression. Deregulation of EGFR signaling is associated with poor prognosis in various tumor types, including breast cancer, head and neck cancer, prostate cancer, non–small cell lung cancer (NSCLC), and GBM (*18*–*23*). There are multiple mechanisms through which EGFR mediates tumor initiation and progression, all of which occur in primary gliomas (Fig. 1). Of these mechanisms, increased EGFR abundance is commonly found in primary GBMs and can occur by gene amplifica-

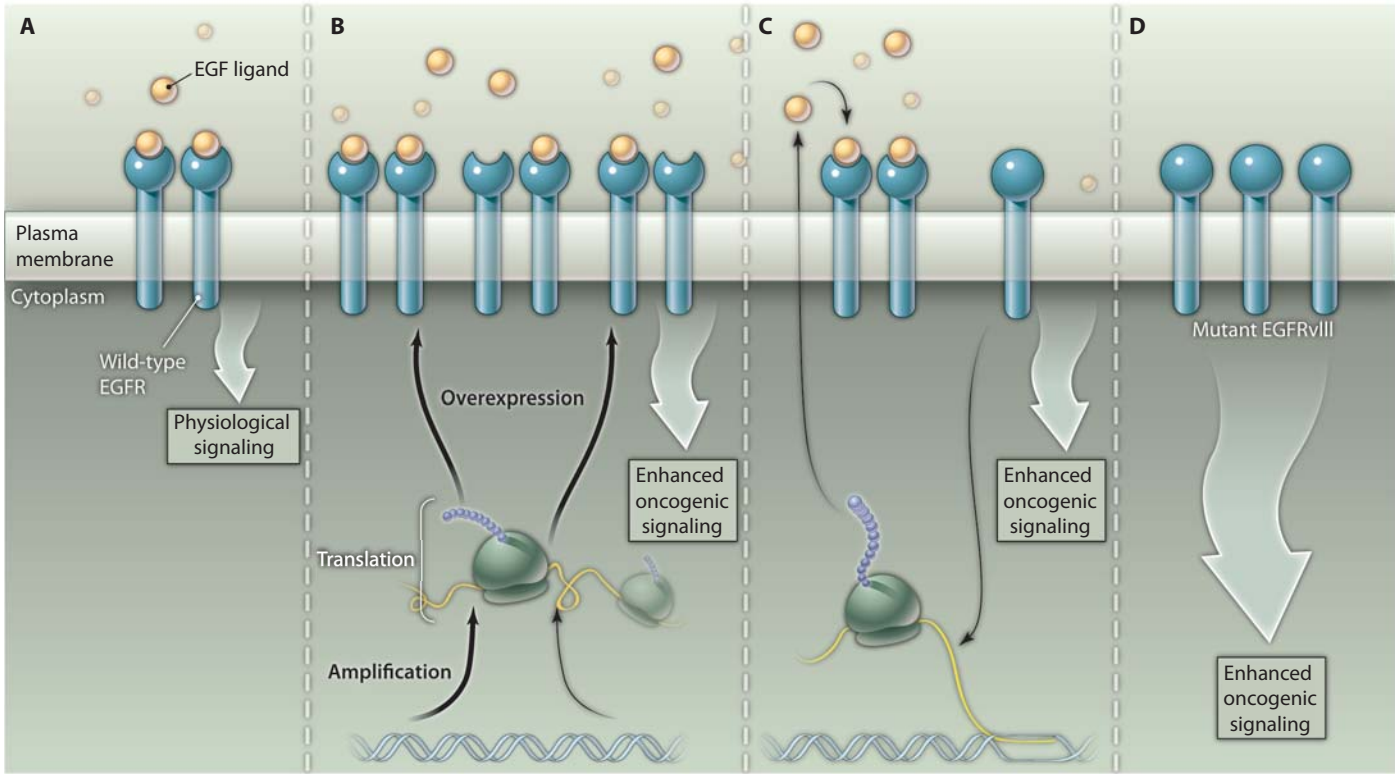

Fig. 1. Mechanisms of EGFR deregulation in glioblastoma. (**A**) EGFR receptor and ligand expression is normally tightly regulated. In primary GBMs, three major mechanisms lead to deregulated oncogenic signaling. (**B**) The most common mechanism involves receptor overexpression or amplification, which results in an increase in competent receptor signaling complexes at the cell surface and enhanced ongogenic signaling. (**C**) Alternatively, oncogenic signaling may arise through the activation of autocrine loops. Secretion of increased amounts of EGF-family ligands either through the action of mutant EGFRvIII or increased matrix protease activity leads to increased wild-type EGFR signaling. (**D**) The third mechanism of signal deregulation is through receptor mutants that result in constitutive receptor activation and diminished turnover. The patterns of signaling activated by these mutant receptors are often distinct from those activated by the wild-type EGFR.

tion, increased translation of the EGFR gene, or both (Fig. 1). EGFR amplification occurs in 40 to 70% of primary GBMs, but is not observed in lower-grade astrocytomas (*19*, *24*). This suggests that EGFR activation may drive tumorigenesis in primary GBMs. All primary glioblastomas with EGFR gene amplification have concurrent EGFR protein overexpression, but only a subset (70 to 90%) of tumors with EGFR protein overexpression also show EGFR gene amplification, indicating that a fraction of GBM tumors show increased receptor abundance in the absence of gene amplification (*25*). Intriguingly, breast cancer patients exhibiting an increase in ErbB2 receptor abundance without changes in gene copy number are more likely to have a clinical outcome similar to that of patients who do not express ErbB2 compared to those with both ErbB2 receptor overexpression and gene amplification (*26*). This finding would suggest that there may be underlying biological and signaling differences between these two routes to receptor overabundance in the context of the ErbB2 receptor in breast cancer (*26*), but it is unclear whether differences in EGFR gene amplification versus protein overexpression have similar effects in GBM.

Autocrine mechanisms of action. EGFR overexpression in primary GBMs is occasionally accompanied by increased abundance of its cognate ligands, EGF and transforming growth factor–α (TGFα). This suggests the existence of an autocrine loop that results in unregulated chronic EGFR signaling (Fig. 1) (*27*). Additionally, a study of 43 brain tumors showed that GBMs have increased abundance of the ADAM12 (a disintegrin and metalloprotease 12) metalloproteinase, leading to the increased cleavage and release of heparin-binding EGF-like growth factor (HB-EGF), an extracellular matrix-bound ligand of EGFR (*28*). Gene expression analysis of U251MG GBM cells expressing the constitutively active EGFR mutant, EGFRvIII, indicates that there is a concurrent up-regulation of TGFα and HB-EGF (*29*). This study also established that an EGFRvIII-HB-EGF-wild-type EGFR autocrine loop is present in U251MG cells, in which EGFRvIII stimulates the secretion of HB-EGF that in turns binds to and activates wild-type EGFR (Fig. 1) (*29*).

Activating receptor mutations. In addition to increases in receptor and ligand abundance, activating mutations of EGFR have also been found in GBMs. The Cancer Genome Atlas (TCGA) consortium identified EGFR as the fourth most highly mutated gene in a compendium of common cancer genes sequenced in a cohort of 91 GBM tumors (*3*). EGFR mutations ranged from extracellular domain point mutations and deletions to deletions in the cytoplasmic tail of the receptor (Figs. 1 and 2) (*30*–*34*).

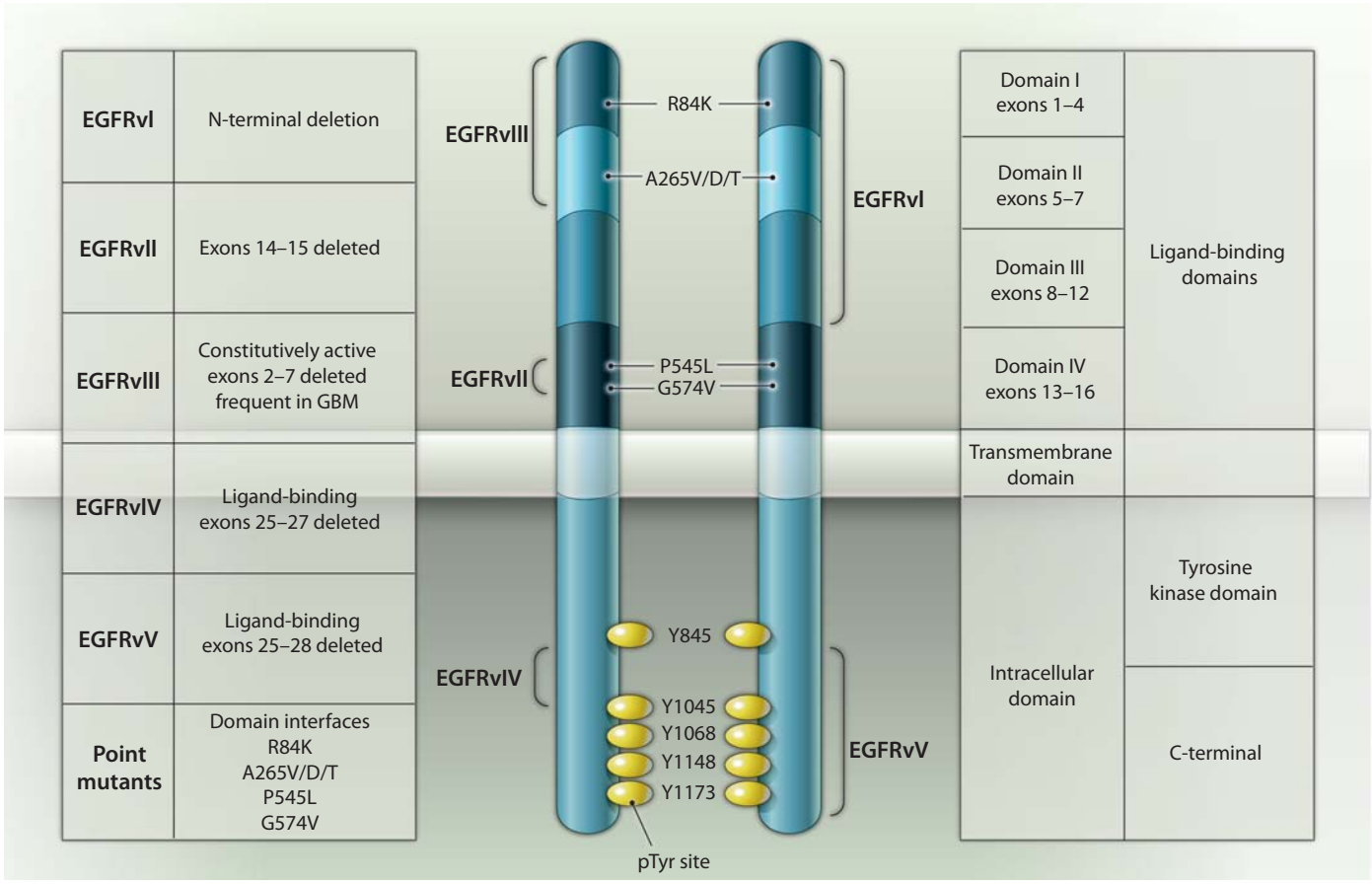

Fig. 2. EGFR mutations in glioblastoma. The six major EGFR mutations that have been identified in GBM are annotated, with their location on the EGFR gene and domain organization. In the open active form of the receptor, domains I and III come together to form the ligand-binding domain. The point mutations R84K, A265V/D/T, P545L, and G574V are located at the interfaces of domains I, II, and IV, which prevents the formation of the closed inactive form and shifts the equilibrium to favor the open form of the receptor. Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; G, Gly; K, Lys; L, Leu; P, Pro; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

A number of deletion mutations that involve the EGFR extracellular domain are exclusively found in GBM; these include the mutants that encode the EGFR type I and type II variants (EGFRvI and vII) (*33*, *34*), which give rise to truncated proteins that are believed to be oncogenic. Point mutations that reside primarily at the interfaces of the various domains in the extracellular region of EGFR are another class of mutations identified in ~14% of GBMs (*31*). These mutations include R84K and A265V/D/T at the domain I/II interface, and P545L and G574V at the domain II/IV interface. These mutations are thought to prevent tethering of domain II and IV, which maintains the closed, inactive form of EGFR and are therefore thought to favor an open, active EGFR conformation (*10*, *11*). Evidence for this mechanism stems from the fact that these mutants are constitutively active but still capable of binding ligand (*31*). EGFR kinase domain mutations commonly found in NSCLC are rare in GBM, whereas extracellular mutations that are common in GBMs are rare in NSCLC (*21*, *31*). However, the molecular basis of the organ site specificity of these mutations and their functional consequences remain unknown.

The cytoplasmic tail deletion mutants EGFRvIV and vV are also found exclusively in GBMs (*30*). These mutations are thought to occur at a low frequency (~15% of EGFR-overexpressing GBMs) and may exhibit defects in receptor internalization. In particular, the EGFRvV mutant lacks a c-Cbl binding site at Y1045 that is required for EGFR ubiquitination and degradation after ligand binding (*35*). EGFRvIV and vV can still bind ligand and have the potential to modulate oncogenic signaling pathways commonly elicited by wild-type EGFR. The ability of these mutants to confer tumorigenicity, and the signaling mechanisms they activate, has yet to be assessed.

The EGFRvIII mutant receptor. The most common and best-studied EGFR mutation found in GBM is the type III EGFR variant deletion mutant (EGFRvIII), which lacks exons 2 to 7 of the EGFR gene (*34*). EGFRvIII is also found in non–small cell lung, breast, and prostate cancers, albeit at much lower frequencies than in GBM (*36*, *37*). Approximately 50 to 60% of GBMs that overexpress wild-type EGFR also express EGFRvIII (*30*, *38*). Moreover, clinical studies have shown a correlation between the presence of the EGFRvIII receptor and poor prognosis in patients with GBM (*39*).

The loss of 801 base pairs in the EGFRvIII deletion mutant excises domains I and II of the extracellular region of wild-type EGFR. Loss of the domain II loop is

thought to prevent formation of the closed inactive conformation, favoring a shift in equilibrium to the open active conformation of the receptor. Although the mechanisms that drive formation of EGFRvIII are not known, the gene deletion may arise from a recombination event between Alu sequences flanking the junctions in introns 1 and 7 of the EGFR gene (*40*).

Although EGFRvIII cannot bind EGFR-family ligands, it is constitutively tyrosine phosphorylated at 10% of the extent of phosphorylation of ligand-stimulated EGFR (*41*). EGFRvIII has been consistently shown to be tumorigenic (*38*–*41*). Inoculation of nude mice with U87MG GBM cell lines expressing EGFRvIII led to faster tumor formation compared to inoculation with parental U87MG cells or cells expressing wild-type EGFR (*42*). Furthermore, EGFRvIII may drive clonal selection, because U87MG-EGFRvIII cells, when mixed with the parental cell line at 1:50,000 ratios in a murine xenograft model, outgrew the parental cell line (*43*). Analysis of proliferation and apoptosis markers indicated that the increase in tumorigenicity resulted from an increase in proliferation and a corresponding reduction in apoptosis (*43*). NR6 murine fibroblast cells bearing EGFRvIII showed increased motility, whereas U87MG cells transfected with increasing amounts of EGFRvIII showed corresponding increases in cell invasion and migration (*44*, *45*).

The transforming ability of EGFRvIII only manifests itself in the context of other genetic mutations. For example, the expression of EGFRvIII alone was insufficient to form high-grade tumors in genetically engineered mouse models (*46*). Only when EGFR was coexpressed in the context of other genetic lesions, such as oncogenic Ras or loss of the tumor suppressor Ink4A/Arf, did tumors form (*46*–*48*). Similarly, reconstitution of the tumor suppressor PTEN (phosphatase and tensin homolog deleted from chromosome 10) into U87MG cell–expressing EGFRvIII led to decreased cell proliferation (*49*). Collectively, this evidence implies that although EGFRvIII is an important driver of transformation in primary GBMs, transformation requires its cooperation with genetic aberrations that occur in other cellular pathways.

Oncogenic EGFR Signaling Networks in GBM

A detailed description of the canonical EGFR signaling networks and their regulation by receptor endocytosis and negative feedback is beyond the scope of this review; readers are referred to recent reviews (*50*, *51*). Here, we focus on deregulated signaling events that occur in EGFRvIII-driven GBM.

Defects in receptor internalization. Activation of the wild-type EGFR leads to its rapid internalization and termination of the signal (*51*), driven by the recruitment of the ubiquitin ligase c-Cbl and Cbl-interacting adaptor, SETA, which are involved in ubiquitin-mediated degradation, through the phosphorylation of Y1045 on the EGFR (*52*). In contrast, EGFRvIII shows defective internalization, resulting in its constitutive localization to the plasma membrane (*41*). It is thought that failure of EGFRvIII to internalize results in sustained, unattenuated EGFRvIII signaling, contributing to its transforming ability (*41*). Evasion of this negative-feedback regulation has been attributed to the low degree of constitutive EGFRvIII phosphorylation, which may be below the threshold required for c-Cbl and SETA binding (*53*). In fact, it has been shown that EGFRvIII is hypophosphorylated at Y1045 and fails to become polyubiquitinated and degraded (*54*, *55*). EGFRvIII is internalized, but at a much slower rate than unstimulated wild-type EGFR (*50*), and the small population of internalized EGFRvIII receptors is not transported to the lysosome for degradation but rather recycled back to the cell surface (*54*). EGFRvIII did not bind to Cbl through its Y1045 site, but rather through an indirect interaction with the growth factor receptor–bound protein 2 (Grb2) adaptor protein (Fig. 3). Cbl binding failed to elicit EGFR ubiquitination under these conditions, resulting in sustained receptor activation (*54*). Overexpression of Cbl in the context of EGFRvIII expression in NIH3T3 cells leads to receptor internalization and degradation (Fig. 3) (*56*). One hypothesis that remains to be explored is whether increasing EGFRvIII activation, perhaps by forcing receptor dimerization, could increase receptor phosphorylation beyond the threshold required for Cbl-mediated polyubiquitination and degradation, ultimately driving signal termination.

EGFRvIII receptor phosphorylation. Mutational analysis of tyrosine phosphorylation sites on EGFRvIII has been performed in an attempt to understand the biological sequelae of EGFRvIII signaling. Mutation of EGFRvIII Y1068, Y1148, or Y1173 leads to a decrease in intracranial tumor volume compared to intact EGFRvIII in U87MG GBM orthotopic xenograft system, implicating these sites in tumorigenicity (*41*). This is in contrast to wild-type EGFR, where only a combination of all three mutations affects transformation and mitogenic potential (*57*). Although the tyrosine sites that are phosphorylated on EGFRvIII are the same as those phosphorylated in wild-type EGFR, quantitative differences in receptor phosphorylation occur between these two receptors. For instance, activation of wild-type EGFR by EGF markedly increases Y1086 phosphorylation (*58*), whereas for EGFRvIII, the total amount of Y1086 phosphorylation remains relatively stable despite increases in mutant receptor abundance (*59*). As a consequence of these quantitative differences in receptor phosphorylation, signaling in response to EGFRvIII is distinct from that in response to activation of the wild-type receptor, resulting in greatly enhanced tumorigenic activity.

The PI3K pathway. In response to growth factor signaling, the class Ia phosphoinositide 3-kinases (PI3Ks) are activated to convert plasma membrane phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] to phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$] (*60*). The formation of $PI(3,4,5)P_3$ triggers the recruitment of a number of signaling proteins through direct lipid binding to the pleckstrin homology (PH) domain, including the kinases phosphoinositide-dependent protein kinase 1 (PDK1) and Akt (*60*). PDK1 phosphorylates Akt on T308. Phosphorylation of Akt by PDK1 on T308 and on S473 by the mammalian target of rapamycin complex 2 (mTORC2) is required for full kinase activity (*61*). Akt phosphorylates a large number of substrates at the consensus RxRxxS/T motif, where x is any amino acid (*62*). This phosphorylation usually leads to the binding to and inhibition of substrate function by the 14-3-3 family of proteins. These substrates play important roles in the regulation of cell survival [Bcl-2/Bcl-XL-associated death promoter (BAD), FOXO transcription factors], growth [tuberous sclerosis complex 2 (TSC2), proline-rich Akt substrate 40 (PRAS40)], cell cycle (p27 and p21 CKIs), and metabolism [glycogen synthase kinase 3 (GSK3)] (*63*).

The PI3K pathway is negatively regulated by phosphophoinositide phosphatases, including Src homology 2 domain–containing inositol phosphatases (SHIP-1 and -2) and

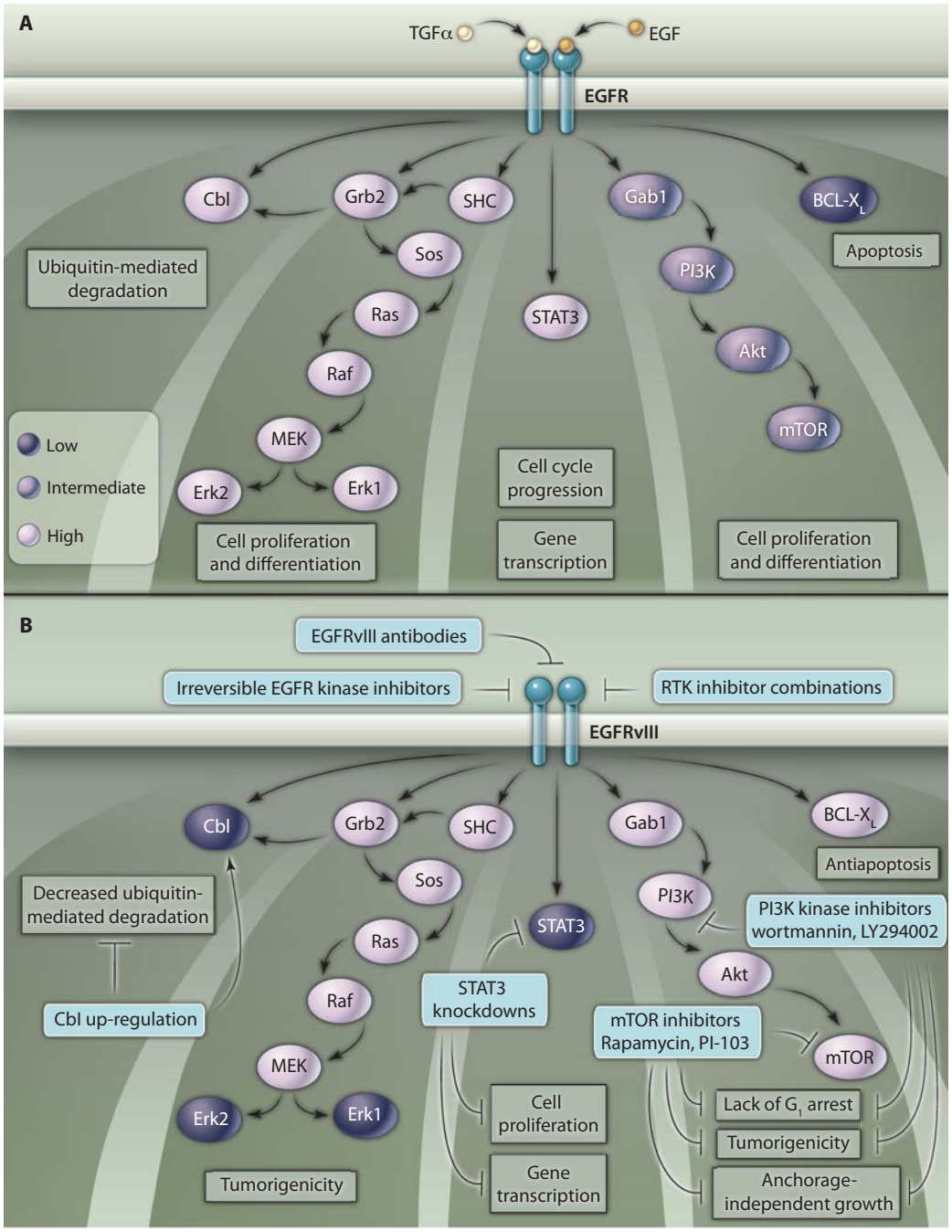

Fig. 3. A comparison of wild-type EGFR and EGFRvIII signaling networks. The major signaling pathways downstream of (**A**) ligand-bound wild-type EGFR under normal physiological conditions and (**B**) constitutively active EGFRvIII in GBM cells include the PI3K, STAT3, MAPK, and BCL-X_L signaling pathways. (A) Binding of EGF or TGFα to wild-type EGFR initiates transient activation of the Ras-Raf-MEK-Erk1/2 pathway, a marked increase in STAT3 phosphorylation, and intermediate activation of the PI3K-Akt-mTOR pathway. Receptor phosphorylation also results in the recruitment of negative regulators such as Cbl that promote receptor internalization and signal termination. (B) Amplification of EGFRvIII also leads to activation of the Ras-Raf-MEK pathway but does not result in high Erk1/2 phosphorylation. Similarly, constitutive activation of EGFRvIII does not increase STAT3 phosphorylation, although STAT3 knockdown in EGFRvIII tumor cells decreases cell proliferation and viability. EGFRvIII activates the PI3K-Akt-mTor pathway to a larger extent than wild-type EGFR. Consequently, EGFRvIII cells are susceptible to PI3K and mTOR inhibitors. EGFRvIII increases BCL-X_L abundance, which confers chemoresistance to classical therapeutic regimens. EGFRvIII shows defective receptor internalization because of decreased Cbl recruitment, and ectopic expression of Cbl in EGFRvIII cells promotes receptor internalization and degradation. The efficacy of EGFR-targeted agents vary, with reversible EGFR kinase inhibitors being less effective than irreversible inhibitors or EGFRvIII-specific antibodies.

PTEN (*60*). The former converts $PI(3,4,5)P_3$ to $PI(3,4)P_2$, whereas the latter converts $PI(3,4,5)P_3$ to $PI(4,5)P_2$. PTEN is a tumor suppressor and is mutated or deleted in a variety of cancers, including in 50% of GBMs (*1*, *25*). Loss of PTEN upsets the PI3K-PTEN balance and results in increased $PI(3,4,5)P_3$ abundance and Akt activity, driving uncontrolled growth and survival.

EGFRvIII activates the PI3K-AKT pathway in fibroblasts, GBM cell lines, and immortalized astrocytes (*64*, *65*). EGFRvIII-expressing NIH3T3 cells had substantially higher PI3K activity than did cells with wild-type EGFR (Fig. 3) (*64*). Furthermore, consistent with constitutive activation of EGFRvIII, increased PI3K activity was independent of EGF. Unlike the ErbB3 receptor, which has multiple docking sites for the SH2 domain of the p85 subunit of PI3K, EGFRvIII likely interacts with the p85 subunit—and activates PI3K—indirectly through their interactions with the Gab1 adaptor. Supporting this notion, antibody to Gab1 immunoprecipitates from EGFRvIII-expressing cells showed increased PI3K activity compared to immunoprecipitates in similar experiments performed in EGF-stimulated wild-type EGFR cells (*64*).

The importance of the PI3K-Akt pathway to the transforming activity of EGFRvIII was emphasized by the decrease in Akt activity associated with treatment of cells with the EGFRvIII inhibitor AG1478,

and the inhibition of anchorage-independent growth by the PI3K inhibitor wortmannin (*64*, *65*). One mechanism through which PI3K may contribute to EGFRvIII-mediated transformation is by decreasing the abundance of the cyclin-dependent kinase (CDK) inhibitor p27. The retinoblastoma protein (Rb) regulates entry into the cell cycle through its ability to bind and inactivate the E2F transcription factor. Upon receiving mitogenic signals, CDK-cyclin complexes form, which hyperphosphorylate Rb and release E2F for the transcription of a large number of genes required for progression into S phase (*66*). The entry and progression into the cell cycle is regulated by CDK inhibitors (CKIs) such as p27. Activation of Akt by PI3K leads to the phosphorylation and consequent nuclear exclusion of members of the forkhead transcription factor family and thereby to a decrease in the production of p27 (*65*). EGFRvIII-expressing U87MG GBM cells have decreased p27 abundance and increased CDK2–cyclin A activity relative to parental cells, leading to hyperphosphorylation of Rb and lack of G_1 arrest under conditions of serum starvation (*65*, *67*). Treatment with the PI3K inhibitor LY294002 or with dominant-negative (DN) Akt restored p27 abundance and G_1 arrest. In addition, DN-Akt decreased the tumorigenicity of EGFRvIII-expressing U87MG xenografts (*65*). Furthermore, an immunohistochemistry study of human GBMs showed a correlation between the presence of the EGFRvIII protein and phosphorylation of Akt, mTOR, and the forkhead transcription factors (*68*).

The Erk1/Erk2 MAPK pathway. Following ligand binding, receptor dimerization, and EGFR transphosphorylation, activation of the MAPK signaling pathway is triggered by Grb2 binding directly to the receptor at Y1068 and indirectly through Src homology domain–containing adaptor protein C (SHC) binding at Y1173 and Y1148 (*69*). Grb2 recruits the son of sevenless (Sos) guanine nucleotide exchange factor to the receptor complex, initiating the MAPK cascade. With EGFRvIII, MAPK cascade activation is sustained, consistent with the constitutive activity of the receptor, and coimmunoprecipitation studies revealed constitutive association of SHC and Grb2 with EGFRvIII at EGFR residues Y1148 and Y1068, respectively (*70*–*73*). EGFRvIII-U87MG cells showed twice the Ras activity of the parental cells (*72*). MEK (MAPK kinase) activity is four times higher in EGFRvIII-expressing fibroblasts than in the corresponding cells expressing wild-type EGFR (*73*). Intriguingly, although EGFRvIII increases activation of the upstream components RAS and MEK, phosphorylation of extracellular signal–regulated kinase 1 and 2 (Erk1/2) does not increase in multiple cell types (*59*, *73*). This suggests that, although EGFRvIII activates Ras-Raf-MEK signaling, there may be negative feedback at the level of MAPK phosphorylation in some cell types. Indeed, treating EGFRvIII-expressing cells with the tyrosine phosphatase inhibitor sodium vanadate increased the amount of phosphorylated MAPK under steady-state serum-free conditions, suggesting that MAPK phosphatases (MKPs) may be responsible for Erk dephosphorylation (*73*) (Fig. 3). A recent temporal gene expression study of wild-type EGFR signaling has identified a number of negative-feedback regulators, including the MKPs, that are transiently up-regulated in response to immediate-early growth factor signaling events (*74*). It is plausible that the constitutive nature of EGFRvIII activation results in a hybrid negative-feedback regulation module in which the MKPs are expressed at stable steady-state concentrations that maintain Erk1/2 signaling activity at a low but constitutive level.

The STAT pathway. The signal transducer and activator of transcription (STAT) proteins are a family of latent transcription factors that are recruited to ligand-bound EGFR dimers through their SH2 domains. The kinase domain of EGFR phosphorylates the STATs and thereby induces their homo- or heterodimerization by means of SH2-phosphotyrosine interactions (*75*, *76*). The dimeric STAT complex translocates to the nucleus, where it binds to specific DNA promoter sequences, recruits other transcriptional regulators, and activates the transcription of multiple genes important for cell survival. Unlike wild-type EGFR, EGFRvIII does not appear to activate the STAT3 pathway through direct phosphorylation (*59*, *77*, *78*). Despite this lack of STAT3 phosphorylation by EGFRvIII, STAT3-null astrocytes carrying EGFRvIII display reduced tumor volume compared to control astrocytes in subcutaneous xenografts (*78*). EGFRvIII interacts with STAT3 both in cytoplasmic and in nuclear fractions (*78*). Screening a panel of human GBM tumors for the presence of EGFRvIII and STAT3 revealed that a small population of primary tumor cells demonstrated co-localization of EGFRvIII and STAT3 in the nucleus (*78*). Nuclear localization of EGFRvIII in GBMs, coupled with evidence that wild-type EGFR translocates to the nucleus where it may play a role in transcription (*79*), raises the intriguing possibility that in addition to its role in signal transduction, EGFRvIII may also function as a transcription factor in glioma cells. Wild-type EGFR has previously been implicated in the activation of the cyclin D1 promoter gene expression in breast cancer cell lines (*80*). Further studies are necessary to determine if EGFRvIII is capable of transactivating gene expression in concert with STAT3 and if developing transactivation antagonists to EGFR is a feasible strategy to subvert EGFRvIII oncogenic effects.

EGFR and Targeted Therapeutics in GBM

The poor prognosis of GBM patients is due in part to classical chemo- and radioresistance properties demonstrated by these tumors. The current standard of care is based on a protocol developed by Stupp (Stupp regimen) (*2*). Newly diagnosed GBM patients undergo surgical resection of the tumor followed by concomitant temazolamide (TMZ) with radiotherapy and subsequent administration of TMZ for 6 weeks as an adjuvant. Because EGFRvIII has been shown to contribute to tumor progression and chemo-radioresistance, an attractive strategy would be to develop targeted therapeutics against this receptor for use as a monotherapy, thereby minimizing the systemic side-effects of whole-brain irradiation and TMZ administration, especially because genetic manipulation of EGFRvIII has resulted in tumor shrinkage in a preclinical setting.

Antibodies directed against EGFRvIII. The deletion of exons 2 to 7 of the EGFR gene results in the formation of a tumor-specific epitope in EGFRvIII, to which monoclonal antibodies have been generated (*81*, *82*). Y10 is one example of an EGFRvIII-specific immunoglobulin G2a (IgG2a) murine monoclonal antibody that recognizes both the human and murine forms of EGFRvIII (*82*). Systemic administration of this antibody led to tumor shrinkage in subcutaneous xenografts of EGFRvIII-expressing B16 melanoma cells in C57BL6/J mice, but had no effect on intracranial tumors in orthotopic xenografts in the same mouse strain. This finding highlights the need to develop therapeutics capable of crossing the blood-brain barrier

(BBB). (*82*). Monoclonal antibody 806 (mAb806) is another example of an EGFRvIII-specific antibody. This antibody recognizes a cysteine loop on EGFR that is exposed on EGFRvIII and a high-mannose form of the wild-type EGFR that occurs only when the wild-type receptor is overexpressed (*83*). Systemic administration of mAb806 to nude mice with intracranial EGFRvIII-U87MG xenografts (Fig. 3) elicited tumor shrinkage and increased survival (from 13 to 21 days) (*81*). mAb806 decreased the volume of tumors carrying EGFRvIII by 65 to 95%, but did not affect parental tumors that lacked EGFRvIII. Tumor shrinkage was attributed to mAb806's ability to prevent EGFRvIII receptor phosphorylation and the consequent activation of downstream signaling pathways while also decreasing the abundance of antiapoptotic protein Bcl-XL, a member of the BCL2 family of antiapoptotic proteins that inhibit mitochondrial outer-membrane permeabilization (MOMP)–induced programmed cell death (*84*). However, durable remissions were not achieved and tumors relapsed after 3 weeks of drug administration (*81*, *85*, *86*). This result led to the development of protocols that involve coadministration of mAb806 with other small-molecule EGFR inhibitors (AG1478) and antibodies to EGFR (mAb528) (*85*, *87*). These treatments had additive effects on tumor shrinkage in established EGFRvIII-expressing subcutaneous xenografts in a therapeutic regimen. A recent Phase I study of mAb806 in eight patients with cancer types at different organ sites demonstrated that it was safe and well tolerated (*88*). mAb806 crossed the BBB and localized to the tumor in a patient with anaplastic astrocytoma (*88*). Although this trial was not intended to show therapeutic efficacy, the ability of mAb806 to cross the BBB and localize to brain tumors holds promise that such EGFRvIII-specific antibodies may someday be used as therapeutic agents, particularly when conjugated to radioisotopes or toxins. It is important to note that the EGFR-specific antibody cetuximab, which is approved for the treatment of colorectal head and neck cancers, has only shown preclinical efficacy in GBM tumor models when administered intracranially and not systemically (*89*, *90*). Intracranial administration of drugs is invasive and increases the risk of infection in GBM patients. The mechanisms by which mAb 806 crosses the BBB have yet to be characterized; understanding this process will be crucial in designing a new generation of EGFR-specific antibodies that are efficacious when administered systemically to high-grade glioma patients.

Small-molecule kinase inhibitors. Small-molecule kinase inhibitors that competitively bind to the adenosine 5′-triphosphate (ATP)–binding pocket in the EGFR kinase domain, thereby reversibly disrupting receptor catalytic activity, present another option for EGFR therapeutics. Because these molecules target the EGFR ATP-binding pocket that shares many common features with other protein tyrosine kinases, they are generally less specific than monoclonal antibodies, which are raised against specific protein epitopes (*91*). Three reversible inhibitors of the kinase activity of EGFR (gefitinib, erlotinib, and lapatinib) have been approved for use in lung and breast cancer patients (*92*). Multiple studies, however, have shown that EGFRvIII-bearing xenograft models are resistant to monotherapy with either erlotinib or gefitinib (*93*–*96*). In one study, administration of the irreversible EGFR inhibitor HKI-272 reduced tumor volume in a mouse model of EGFRvIII-driven NSCLC (*93*), suggesting that irreversible inhibition of EGFRvIII may represent a strategy to overcome resistance to the inhibition of EGFR catalytic activity (Fig. 3). Similar to the data garnered from preclinical studies, several trials have shown that the administration of gefitinib, erlotinib, or lapatinib as a monotherapy does not offer an advantage over standard treatment regimens such as the Stupp protocol in the clinical setting (*97*–*100*).

A retrospective study of 49 recurrent GBM patients treated with EGFR kinase inhibitors revealed a correlation between coexpression of EGFRvIII and PTEN and favorable response to treatment with inhibitors of EGFR activity (*95*). PTEN inhibits PI3K signaling, and loss of PTEN function results in increased $PI(3,4,5)P_3$ abundance and increased Akt activity. One mechanism whereby loss of PTEN could result in EGFR inhibitor resistance might be through uncoupling of the PI3K-AKT-mTOR pathway from EGFRvIII oncogenic signals, such that inhibition of EGFR activity using targeted therapeutics does not diminish the activation status of the PI3K-AKT-mTOR pathway (*101*). This mechanism is supported by the finding that the phospho-AKT status of EGFRvIII, PTEN-null U87MG cells is unaltered by the administration of erlotinib (*95*). The correlation between PTEN and EGFRvIII is meaningful because PTEN is mutated or deleted in 50% of GBM patients (*25*, *59*). The combination of erlotinib with inhibitors of the PI3K-AKT-mTOR signaling pathway resulted in a cooperative increase in cell-cycle arrest of EGFRvIII-positive, PTEN-null GBM cells (*49*, *102*), suggesting that inhibition of both the PI3K-AKT-mTOR pathway and EGFRvIII is required to achieve a full cytostatic response, presumably as a result of PI3K-independent pathways activated by EGFRvIII.

A recent study showed that the phosphorylation status of mTOR and its downstream target rpS6 were accurate biomarkers for an antiproliferative response to the EGFR inhibitor erlotinib in glioma cells with wild-type EGFR (*103*). Following erlotinib administration, Akt phosphorylation in PTEN-mutant glioma cells decreased, despite their lack of phenotypic response (cell viability) to treatment, suggesting that Akt phosphorylation is not predictive of GBM cell viability in response to erlotinib. Consistent with this observation, pharmacological inhibition of Akt or genetic knockdown of Akt isoforms had no effect on glioma cell viability. Similarly, a constitutively active form of Akt had no effect on the antiproliferative response of glioma cells to erlotinib. Rather, protein kinase C–α (PKC-α) acted as a signaling intermediate between wild-type EGFR and mTOR. PKC-α is a serine-threonine kinase that is activated by association with diacylglyerol (DAG) and Ca^{2+} as well as phosphorylation by PDK1. Upon EGFR activation, phospholipase C–γ (PLC-γ) is recruited via its SH2 domain to the phosphorylated receptor where it is activated and cleaves $PI(4,5)P_2$ into IP_3 and DAG. Production of DAG leads to PKC activation and phosphorylation of downstream targets that include RAF1 and GSK3β (*104*). Using a pan-PKC inhibitor, the authors demonstrated that decreased tumor cell viability was observed regardless of EGFR or PTEN protein abundance status, suggesting that PKC inhibitors may provide alternatives for patients with mutant PTEN who are refractory to EGFR monotherapy.

Studies using phosphoproteomic approaches (*59*, *105*, *106*) have recently demonstrated that multiple receptor tyrosine kinases (RTKs), including EGFR, are coactivated in GBM cell lines and tumors. RTK coactivation is an alternative mechanism by which GBM

mediates chemoresistance to EGFR inhibitor monotherapy. Although in vitro studies suggest that inhibition of individual RTKs is insufficient to eliminate downstream oncogenic pathways, chemical inhibition or genetic depletion of multiple RTKs [such as c-Met and platelet-derived growth factor receptor (PDGFR)] in combination with EGFRvIII inhibition led to enhanced U87MG GBM cell death (Fig. 3) (*59*, *106*). Overcoming chemoresistance by simultaneously targeting multiple RTKs appears to be independent of PTEN status, suggesting that GBM tumors achieve chemoresistance through multiple mechanisms. Although these studies have provided insight into the contribution of RTK coactivation to GBM chemoresistance, the mechanisms underlying this process and the integrated signaling networks that result from the activation of multiple RTKs remain poorly understood. Further research into this burgeoning field will allow physicians to tailor treatment protocols to achieve maximum efficacy in tumors driven by different RTK coactivation combinations.

EGFR and Integrative Systems Biology

Many questions remain regarding EGFR signaling in GBM. For instance, how are intracellular signaling networks affected by GBM-specific EGFR mutations? How is information processed from coactivated RTKs to downstream phenotypes? Can the response to chemotherapy be predicted from specific biomarkers? Our understanding of glioma biology has benefited from the availability of information derived from the application of multiple "-omic" approaches to GBM cell lines and tumors, including genome-wide sequencing (*3*, *4*), genome-wide copy number (*3*, *107*, *108*), and transcriptomic (*109*), epigenetic (*110*, *111*), and phosphoproteomic analysis (*59*, *105*, *106*). In addition, several studies have provided information on specific aspects of EGFR signaling, such as in vitro interactome and domain-binding (*16*, *69*). This knowledge base will likely increase exponentially with new advances in analytical technologies such as next-generation deep sequencing approaches and high-throughput bead-based phosphoproteomic measurements (*105*, *112*). The challenge will then shift to integrating disparate information types to enable a better understanding of key regulatory points within the GBM signaling networks. Fortunately, this challenge is not unique to GBM, and initial attempts at integrating multiple data sets in other biological contexts have yielded insights into tumor biology. Here, we summarize how systems biology has led to insights into multiple aspects of EGFR biology. The application of these systems-biology approaches to EGFR signaling in GBM will enable the identification of novel therapeutic targets and therapeutic regimens (Fig. 4).

ErbB-family signaling in glioblastoma. Although much of the previous work on GBM biology has focused on the EGFR, as the prototypical member of the ErbB family, large-scale studies of GBM tumors and cell lines have revealed that other members of this receptor family may also play important roles in glioma (*3*, *106*). For instance, cancer genome sequencing has uncovered point mutations in the ErbB2 receptor (*3*). Although this study did not delve into the

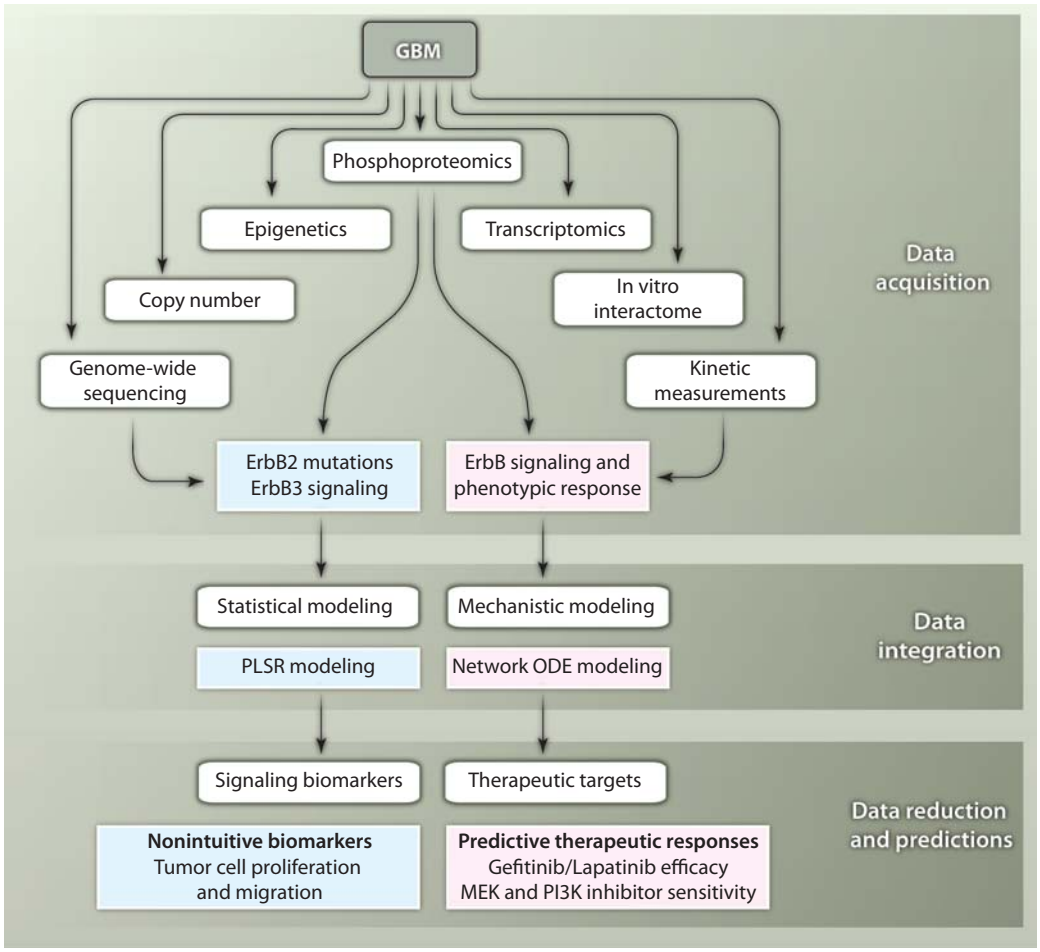

Fig. 4. Integrative network biology in glioblastoma. Information on the EGFR signaling network information can be acquired with various different approaches, each providing orthogonal information about the EGFR system. By integrating this data with computational modeling, it is possible to gain mechanistic insights into EGFR signaling. The figure depicts two different approaches to modeling, statistical modeling exemplified by PLSR modeling of the EGFR-ErbB2 signaling network shown in blue (*115*, *117*) and mechanistic modeling of the same network using ODE-based models as illustrated in red (*119*, *120*). Data reduction using statistical methods will generate nonintuitive hypotheses correlating critical signaling components with cancer phenotypes that may serve as biomarkers for tumor cell proliferation or migration. Additionally, sensitivity analysis using ODE-based mechanistic models will identify predict network components that are sensitive to therapeutic intervention.

functional importance of these ErbB2 mutants, these point mutations are reminiscent of those found in the extracellular domain of EGFR (Fig. 2), indicating that ErbB2 may play a role in disease progression in GBM patients. Phosphoproteomic studies using antibody arrays have identified the ErbB3 receptor as a commonly phosphorylated member of RTK coactivation networks in glioma cell lines and tumors (*106*). Because members of the ErbB family form various homo- and heterodimeric combinations, and each dimer pair shows distinct signaling and phenotypic properties, it will be crucial to determine the composition of ErbB dimers present in glioma to ascertain their contribution to disease progression (Fig. 4).

Mathematical modeling. Mathematical modeling approaches have shed light on multiple aspects of ErbB receptor downstream signaling and cellular outcomes. Studies using mechanistic models of receptor dimerization and internalization based on cellular signaling events or biological phenotypes have demonstrated that the particular combination of ErbB receptors at the cell surface influences downstream signaling and phenotypic outcomes in human mammary epithelial cells (HMECs) (*113*, *114*). For example, contrary to the prevailing view, both EGFR and ErbB2 exhibit the same intrinsic ability to activate the ERK pathway (*114*). Modeling showed that sustained ERK activation following EGFR-ErbB2 heterodimer formation is not due to differences in intrinsic pathway activation, but is instead a function of reduced internalization rate and slower degradation of this receptor pair compared to that of EGFR-EGFR homodimers. A similar study included the ErbB3 receptor as a species in the kinetic model and used the same approach to predict the effects of dimerization between ErbB2 and EGFR or ErbB3 on biological phenotypes (*113*). Experimental analyses showed that ErbB2-ErbB3 heterodimers were the preferred dimeric species in cells expressing all three types of ErbB receptors and predicted that cells coexpressing EGFR with small amounts of ErbB2 and ErbB3 will display the same potent phenotypic outcome as cells coexpressing EGFR with amplified amounts of ErbB2. This has important implications for GBM tumors, as the TCGA gene sequencing established ErbB2 as the fifth most commonly mutated gene in GBM tumors, but copy number and gene expression analysis indicate that it is not amplified in these tumors (*3*), unlike subsets of breast and ovarian cancers where the ErbB2 gene is commonly amplified. In some GBM tumors, mutant ErbB2 receptors were coexpressed with amplified EGFR (*3*). The above-mentioned modeling studies suggest that such tumor cells may exhibit a similar oncogenic activity profile as ErbB2-amplified breast cancers if they concurrently coexpress ErbB3 in low abundance. Adapting these mathematical approaches to GBM will not only provide a better understanding of the role of ErbB2 signaling in glioma biology, but may also be used to model the biological and signaling impact of using ErbB-targeted therapeutics in GBM patients.

In addition to mechanistic models, data-driven statistical models have been applied to integrate signaling network information that may yield nonintuitive hypotheses of ErbB signaling. Specifically, partial least-squares regression (PLSR)-based statistical modeling has been used to determine how downstream processes are governed by activation of the EGFR-ErbB2 signaling network (Fig. 4). PLSR was used to correlate phosphoproteomic data obtained by mass spectrometry to phenotypic measurements to determine the signaling nodes that best describe HMEC growth and migration following EGFR and ErbB2 stimulation with EGF and heregulin (*115*). For instance, Annexin II, a target found in the study to strongly correlate with migration, has since been shown to contribute to the migration and invasion in cancer cells (*116*). A reduced model in which only a fraction of phosphorylation sites are used to generate the original PLSR model would be beneficial when full network information is unavailable. Model reduction was performed through a variable importance of projection (VIP) score to determine the EGFR signaling components that were most strongly correlated with HMEC cell growth and migration (*117*). This led to the identification of nine phosphorylation sites on six proteins in the signaling network that were capable of fully recapitulating the predictive power of the full PLSR model. These six proteins included known regulators of cellular proliferation [SHC and Src homology 2 (SH2) domain–containing inositol phosphatase 2 (SHIP-2)] and migration-activated cdc-42–associated kinase (ACK), as well as previously undescribed regulators of cellular proliferation and migration such as the transferrin receptor and solute carrier protein 38 (SCF38). It is not yet known whether these nine phosphorylation sites on these six proteins are predictive of cellular phenotypes only in the context of mammary epithelial cells, but a recent study using similar PLSR approaches in various epithelial cell lines suggested that such computational predictions may be broadly applicable to multiple cell types as a result of common effector processing (*118*). Such approaches can also be applied to the study of EGFR-ErbB2 and EGFR-EGFRvIII signaling networks in GBM; model reduction in this manner may be able to identify nonintuitive therapeutic targets or signaling biomarkers for disease.

Developing therapy through ErbB network modeling. There is an increasing awareness that combinatorial inhibition of multiple components of the EGFR network may be required to overcome resistance to monotherapy (*78*). It would be ideal to be able to predict optimal combinations for treatment in silico before experimental validation in vivo. One way of achieving this is through the use of ODE (ordinary differential equation)–based mechanistic models. Several ErbB-specific network models based on EGF and heregulin activation have recently been constructed (*119*, *120*). In silico perturbations using small-molecule kinase inhibitors have led to experimentally validated predictions of downstream signaling nodes (*119*, *120*). For instance, one such mechanistic model demonstrated that the Akt pathway in EGF-stimulated A431 epidermal cancer cells was more susceptible than Erk to inhibition with ErbB inhibitors gefitinib or lapatinib (*120*). A similar study used an ErbB network model to show that the Erk pathway in heregulin-stimulated cells is less sensitive than that in EGF-stimulated cells to MEK inhibition with U0126 (*119*). The ability of such modeling approaches to predict signaling outcomes upon therapeutic intervention holds promise for their utility in understanding GBM biology. For such ODE models to be applicable to glioma, they must first be adapted to reflect the signaling connectivity, protein expression levels, and mutations implicit in glioblastoma cells. Once these models have been refined, candidates for targeted therapy may be identified by sensitivity analysis of specific or multiple nodes in the EGFR model to determine fragile points in the network that may act synergistically to shut down the critical downstream EGFR pathways governing tumor cell growth and chemoresistance.

Outlook. Through the efforts of the TCGA consortium and other research groups, a comprehensive portrait of the genomic and copy number changes that occur in GBM now exists (*3*, *4*). These studies have demonstrated that genomic aberrations in GBM are dispersed across multiple signaling networks rather than clustered within specific signaling pathways. Integrative systems biology approaches have largely been restricted to in vitro cell line models, primarily as a result of the paucity of large-scale signaling data from patient samples. With the advent of improved phosphoproteomic technologies such as mass spectrometry (*59*), antibody microarrays (*106*), and antibody-conjugated bead-based systems (*105*), it is now possible to quantify phosphorylation networks in tumors with enhanced sensitivity and higher throughput. To translate our knowledge of in vitro GBM biology to the clinic, we propose that it is necessary to apply these tools to analyze and integrate aberrant tumor signaling networks with genomic information from the same tumor samples. For instance, by taking a snapshot of the signaling events that occur in tumors that are positive for GBM-specific EGFR mutations, one can gain an understanding of how downstream intracellular signaling networks are modulated as a result of such mutations. Similarly, by integrating the signaling changes with the genomic alterations in matched tumor pairs from the same patient before and after treatment, and subsequently correlating these data to patient response and prognosis, we will have a better grasp of the signaling mechanisms by which GBM tumors acquire chemoresistance. We believe that the inclusion of network-based signaling data into future genome-wide GBM tumor studies will bridge our knowledge from gene to function and ultimately provide a more complete molecular understanding of this disease.

Conclusion

A decade of biochemistry and molecular biology has led to many seminal findings, including many of the genes implicated in GBM initiation and progression. These data will provide the framework from which to apply high-density network data sets and computational approaches to understand how the multifaceted hallmarks of GBM such as rampant proliferation, diffused invasion, and chemoresistance are governed by EGFR signaling networks. New insights into glioma biology gained from these integrative approaches will undoubtedly generate new avenues for drug and biomarker development to combat this devastating disease.

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