EGFR-dependent Mechanisms in Glioblastoma: Towards a Better Therapeutic Strategy

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EGFR-DEPENDENT MECHANISMS IN GLIOBLASTOMA: TOWARDS A BETTER THERAPEUTIC STRATEGY

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Abstract

Glioblastoma is a particularly resilient cancer: therapies have to be able to reach the brain crossing the Blood Brain Barrier (BBB) and they have to deal with a highly invasive tumor that is very resistant to the damage in the DNA. It seems clear that in order to kill aggressive glioma cells, more efficiently and with fewer side effects on normal tissue, there has to be a shift from classical cytotoxic chemotherapy to targeted therapy. Since the epidermal growth factor receptor (EGFR) is altered in almost 50% of glioblastomas, it represents one of the most promising targets. In fact it has been associated with several steps in tumorigenesis, from tumor initiation to tumor growth and survival, and also with the regulation of cell migration and angiogenesis. However, EGFR kinase inhibitors have produced poor results in clinical trials in this type of cancer, with no clear explanation for the tumor resistance observed. Here we will revise what we know about the molecular function of EGFR in cancer, and in particular in gliomas. We hope to come out with an operational definition of EGFR addiction in certain glioblastomas that could improve the design of future therapies.

Keywords: Glioblastoma, EGFR signaling pathway, EGFR stability, therapy resistance

Introduction

Glial tumors are primary tumors that resemble astrocytes and/or oligodendrocytes. Grade IV astrocytomas (known as glioblastomas (GBM)) are the most common glial tumors and they have a terrible prognosis with a median survival rate of 12 to 15 months. The main features of this type of cancer include high levels of mitosis, diffuse infiltration, a tendency for necrosis, significant angiogenesis, resistance to apoptosis, and widespread genomic aberrations (1). Current standard treatment consists of surgery followed by radiotherapy and cytotoxic chemotherapy with the alkylating agent Temozolomide (2) although treatment remains palliative for most patients. Another important feature of GBM is the high degree of intra and intertumoral heterogeneity. For decades they have been classified as primary GBM (occurring without evidence of a pre-existing, less malignant lesion) that comprises more than 90% of the cases, and secondary GBM (occurring through the progression from a lower grade glioma) that generally affects younger patients (3). However there are not histological differences between these two entities. More recently large scale expression profiles of glioblastomas have provided a molecular classification into three (4) or four (5) main subtypes, with potential implications for patient prognosis and management.

Multiple alterations in the expression level of genes and/or proteins have been identified in glioblastomas, including activation of oncogenes and/or silencing of tumor-suppressor genes. Based on copy number and expression analyses as well as DNA sequencing, researches have confirmed three signaling pathways commonly disrupted in GBM: (i) receptor tyrosine kinases (RTK)/Ras/phosphoinositide 3-kinase (PI3K) pathway (which includes alterations in EGFR (amplified and/or mutated in 40% of cases), the PI3K inhibitor: *phosphatase and tensin homolog (PTEN)* (inactivated in 36% of cases) and the Ras inhibitor *neurofribromatosis 1* (*NF1*) (inactivated in 23% of cases); (ii) the p53 pathway, where the gene *TP53* is mutated in 35% of cases; (iii) the Rb pathway, where the cell-cycle inhibitors *CDKN2A* (*p16INK4A*) and *CDKN2B* are alternatively inactivated in about 50% of cases (6;7). A relatively high frequency of mutations was also found in the *isocitrate dehydrogenase 1 and 2* (*IDH1/2*). However these mutations are preferentially associated with secondary GBM (80% of the cases) with a significant pathogenic role (3). By contrast primary GBMs are characterized by a high proportion of mutations and/or overexpression of *EGFR* gene. *EGFR* codes for a 170kDa glycosylated receptor with tyrosine kinase activity. Altered EGFR function has been associated with GBM tumor initiation and growth, as well as with cell invasion, angiogenesis and resistance to chemo- and radiotherapy (8;9). However, although EGFR kinase inhibitors are useful in treating other types of tumors, they offer poor outcomes in GBM patients. Moreover, contrary to what could be expected, there is some controversy about the correlation between the EGFR amplification and overexpression, and the clinical response to EGFR kinase inhibitors in GBM patients (10-13). These results underline the special nature of the EGFR oncogenic network in these neoplasms. In a simplified view EGFR activates the mitogen activated protein kinase (MAPK) and PI3K signaling cascades, resulting in changes in cell growth, survival, migration and angiogenesis. However the first one responds to many other molecules present in GBMs, and the second one is usually activated by mutations in these tumors. Moreover accumulating evidences suggest that EGFR and its mutant forms regulates other aspects of cancer cell biology like cell

metabolism and response to DNA damage, directly linked to the survival of the cells in the GBM tumorigenic context, that could explain the tumorigenic potential of EGFR in these tumors.

Recent evidences also suggest that in some cases EGFR actions do not depend on its kinase activity. This atypical mode of EGFR signaling could contribute to the failure of the majority of EGFR-targeted agents designed to inhibit its kinase activity.

Here we will try to draw a comprehensive picture of EGFR signaling (in particular those aspects linked to cell proliferation and survival), based on classical models and more recent findings, in order to explain its oncogenic action in aggressive gliomas. We hope that this review will shed some light into the development of targeted therapies for EGFR-dependent GBMs and will suggest better synergistic approaches and/or possible predictive markers.

EGFR structure and mutations in GBM

Epidermal growth factor (EGF) was identified by embryologist Stanley Cohen in the early 1960s and its receptor (EGFR), which is also known as HER-1 or c-erbB-1, was identified a decade later (14). EGFR is one of the four transmembrane growth factor receptor proteins (c-erbB) that share similarities in structure and function. Other members of the c-erbB group include HER2 (c-erbB-2), HER3 (c-erbB-3), and HER4 (c-erbB-4). EGFR is the receptor for members of the EGF-family of extracellular protein including EGF, transforming growth factor-α (TGF-α); amphiregulin (AR), betacellulin , epiregulin and Heparin-binding EGF-like growth factor (HB-EGF)(15). EGFR consists of a single polypeptide chain of 1186 amino acids with three main regions: an extracellular (EC) receptor domain, a transmembrane region (TM), and an intracellular domain (IC) with tyrosine kinase (TK) function (Figure 1). The EC amino-terminal end can be divided into four domains with the L1 and L3 responsible for ligand binding. Cysteine-rich (CR) domains 1 and 2 contain N-linked glycosylation sites and disulfide bonds that determine the tertiary conformation of the external portion of the molecule. A large loop that protrudes from the back of CR2 makes a molecular contact with the respective domain of the other receptor (16). EGFR can form homo- and hetero-dimers with other members of the c-erbB family, resulting in differences in ligand affinity and downstream signaling (15). Dimer formation between two EGFR molecules in the phosphorylation of the tyrosine (Y) residues in the C-terminal tail segment, which serve as docking sites for signaling molecules that contain Src homology domain 2 (SH2) or phospho-tyrosine binding (PTB) domains and are responsible for onward transmission of the signal. The kinase activity of EGFR is stimulated by ligand engagement in a manner that depends on intermolecular interactions (17). In contrast to other kinases the trans-phosphorylation of the activation loop is not a critical event for EGFR activation (18). In fact, recent structural studies have revealed that the EGFR tyrosine-kinase domain has two different conformations. In the inactive one it is able to inhibit its own activity but after EGF induced dimerization, the increase in the local concentration of the kinase domain provokes an allosteric change that forces the activation of EGFR (19;20).

Ligand binding also results in rapid internalization of activated receptors and targeting of internalized EGF-receptor complexes to lysosomes for degradation. The customary EGFR signal attenuator is the ubiquitin-ligase CBL, which binds to the regulatory domain (REG) at the

receptors´ s tail. When bound; CBL attaches mono-and di-ubiquitin moieties to multiple lysine residues of EGFR, thus tagging the receptor for lysosomal degradation. Acceleration of internalization and lysosomal targeting leads to receptor down-regulation which serves to decrease the number of activated receptors in the cell and prevent excessive signaling (see below).

There are several mechanisms that could justify the activation of EGFR signaling pathway in GBM. Overexpression on its own could provoke a local accumulation of the kinase domain that would lead to its activation. Moreover high expression of EGFR ligands has been reported in high grade gliomas (21-23) and there are reports of *TGFα* amplifications, mainly in recurrent gliomas (24). However it is also well known that many of the GBMs with *EGFR* amplification have *EGFR* mutations (16;25). The most common *EGFR* mutations found in GBMs are deletion mutations (EGFRvI to EGFRvV), and in-frame deletion of regions of the extracellular domain like EGFRvIII (present in 30 to 40% of GBMs). A recent massive sequencing of the receptor in GBM tumors has uncovered several oncogenic missense point mutations in the extracellular domain of the receptor that in some cases have been shown to confer oncogenic potential (26), presumably by promoting receptor dimerization. Another common mutation is a truncation of the intracellular region at amino acid 958, EGFRvV, present in 15% of GBMs with *EGFR* amplification. This mutant receptor is internalization-deficient and therefore displays increased ligand-dependent kinase activity (16;25). Mutations of the intracellular portion of *EGFR* are more common in other neoplasms. In fact, the tyrosine kinase domain mutations that have been shown recently to be responsive to specific inhibition of EGFR in lung cancer (27), have not been found in GBMs (28). Of note, multiple mutations are sometimes seen in the same amplified *EGFR* gene, a finding unique to GBM (29). A recent genomic study has revealed the presence of recurrent in frame fusions involving *EGFR* (in 7.6% of GBMs), with the most recurrent partners being *septin 14* (*SEPT14)* and *phosphoserin phosphatase* (*PSPH*). Interestingly the *EGFR-SEPT14* fusions confer mitogen-independent growth, constitutively activate signal transducer and activator of transcription 3 (STAT3) signaling and impart sensitivity to EGFR kinase inhibitors (30).

EGFRvIII accounts for 60 to 70% of EGFR mutations in GBM and involves exons 2 to 7 of the extracellular domain (Figure 1). Regions of multiple Alu repeats in introns 1 and 7 may play a role in mediating susceptibility to this specific gene rearrangement (29). This mutated gene encodes for a receptor that lacks amino acids 6 to 273 and creates a novel tertiary conformation of the extracellular domain. This mutated form resembles the viral EGFR homologue, v-erbB, which exists primarily in dimers, leading to constitutive kinase activation and subsequent oncogenicity (31). The study of several mouse glioma models has demonstrated that the vIII variant is more tumorigenic that the wild type receptor (see below). Although EGFRvIII is unable to bind to its ligands it has been proposed that its oncogenic action is due to a constitutively active kinase activity. In fact the altered kinetics of EGFRvIII could result in a distinct set of downstream signals compared to the wild type EGFR. There are reports of selective and/or constitutive activation of several pathways: PI3K pathway (32;33), Ras (34), c-jun N-terminal kinase (JNK) (35), Src family kinases (SFK) (36), urokinase-type plasminogen activator receptor (uPAR) (37) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (38). Moreover EGFRvIII confers drug resistance to GBM cells through the modulation of B-cell

lymphoma-extralarge (Bcl-XL) and caspase 3-like proteases (39). Some recent transcriptional and biochemical studies have reinforced the different signaling networks activated by wild type EGFR (EGFRwt) and its variant EGFRvIII (33;40;41) which could explain the differences in the tumorigenic potential of both receptors. Intriguingly, the oncogenic capacity of EGFRvIII may propagate into non-expressing cells by means of cell-to-cell transfer of microvesicles (also called exoxomes) (42). Exosomes are membrane-enclosed vesicles, of 30 to 100 nm in diameter, that are derived from endosomes during the formation of multivesicular bodies (43). In GBM it has been shown that exosomes can mediate the horizontal transfer of EGFR (both protein and mRNA) and alter the proliferation of receptor cells (44). Additionally it has been reported that EGFR ligands can accumulate in exoxomes from cancer patients which could contribute to the dialogue between the different tumor cells, and also between the tumor cells and their niche (45). This exoxome-mediated communication could add more complexity to the EGFR-related gliomas.

EGFR kinase-dependent downstream signaling

EGFR signaling is activated by a three step mechanism. First, the binding of any of the specific ligands to the receptor induces dimerization of the ligand-binding domains. Second, the dimerization results in the auto-phosphorylation of five specific tyrosine residues in the carboxy-terminal end of the intracellular part of EGFR: Y992, Y1045, Y1068, Y1148 and Y1173, with Y1173 as the major auto-phosphorylation site (46;47). Third, activated EGFR recruits several signaling molecules that associate with the phosphorylated tyrosines through their SH2 and PTB domains and in many cases become phosphorylated by the receptor. These are the main cascades linked to this EGFR tyrosine kinase activation (Figure 2):

MAPK cascade. EGFR has been classically associated with cell proliferation control through the recruitment of Growth factor receptor bound protein 2 (Grb2) and the activation of the MAPK/ERK (Extracellular signal-regulated kinase). Grb2 recruits the guanine nucleotide exchange factor son of sevenless (SOS) via its SH3 domain, and promotes binding of GTP to Ras, than then binds and activates the RAF kinase. Activated RAF in turn binds to and phosphorylates MEK, which then phosphorylates ERK1/2. Upon activation, ERK kinases can translocate to the nucleus and activate several transcription factors (TFs) including Elk-1 (ETS domain-containing protein), peroxisome-proliferator-activated receptor γ (PPARγ), signal transducer and activator of transcription 1 and 3 (STAT1 and STAT3), C-myc and activating protein-1 (AP-1). Activation of these factors leads to an increased transcription of genes involved mainly in cellular proliferation (48).

Expression of MAPK and activated phospho-MAPK was significantly correlated with proliferation and shorter survival time in gliomas (49). However, while constitutively activated, mutated forms of Ras are found in almost 50% of all human tumors, few Ras mutations have been found in gliomas. However the GTPase activating NF1, which inhibits Ras, is inactivated in 23% of cases (7). Nevertheless, even in *NF1* wild type tumors, high levels of active Ras-GTP are found (50) suggesting that the main mechanism for MAPK-dependent mitogenic signaling in GBM must be mediated by the inappropriate activation of EGFR and/or other membrane

molecules: receptor tyrosine kinases (RTKs), integrins, vascular endothelial growth factor (VEGF).

PI3K signaling. EGFR can modulate the balance between senescence and apoptosis through the recruitment of the p85 subunit of PI3K, and the subsequent activation of the p110 subunit. PI3K phosphorylation of phosphatidylinositol-4, 5-bisphosphate (PIP_2) yields the second messenger phosphatidylinositol (3, 4, 5)-triphosphate (PIP_3). PIP_3 serves as a membrane-docking site for the serine/threonine protein kinase AKT, which binds to PIP_3 with high affinity through its pleckstrin homology (PH) domain. PIP_3 is dephosphorylated to yield PIP_2 by the tumor suppressor protein PTEN, which attenuates AKT signaling. Phosphorylated AKT appears to be able to prevent programmed cell death through targeted inhibition (phosphorylation) of Bad (a pro-apoptotic member of the Bcl-2 family) and caspase-9, and activation of murine double minute 2 homolog (MDM2) and Inhibitor of nuclear factor kappa-B kinase subunit alpha (IKKα) (51). The activated IKKα, in turn, phosphorylates inhibitor of κB (IκB), targeting it for ubiquitination and proteosomal degradation and leading to the activation and nuclear translocation of NF-kB. NF-κB plays and important role in inflammation and cancer and it can induce pro-survival genes like Bcl-XL or caspase inhibitors (52). Activated AKT also promotes cell growth through the activation of mammalian target of rapamycin (mTOR), a master integrator of growth factors signals and nutrient and ATP sensing (53). The downstream effectors of mTOR are eukaryotic initiation factor 4E (eIF4E) and the ribosomal protein S6 kinase (S6K1/2). By the first mechanism, eIF4E binding protein 1 (4E-BP1) suppressor protein factor is phosphorylated and inactivated, whereas by the second mechanism, translation of mRNA by phosphorylation of S6 is enhanced (54).

Elevated phosho AKT levels have been observed in up to 85% of GBM cell lines and patient samples (55). Activation of the PI3K pathway is significantly associated with increasing tumor grade, decreased levels of apoptosis, and with adverse clinical outcome in human gliomas (56). In fact AKT activation correlates with EGFR amplification (57). However mutations in the PI3K/AKT signaling pathway are frequent in GBM suggesting that in many tumors AKT activity would not be sensitive to EGFR inhibitors (see below). Alterations in this pathway include activating mutations and amplifications of *p110*α (6;58;59), and *p110*δ (60), and also gain of function mutations in the *p85*α regulatory subunit (6;61). Moreover, as we have already mentioned, the *PTEN* gene is lost, mutated or epigenetically silenced in 40% -50% of gliomas, resulting in high levels of PI3K activity and downstream signaling (6;7;51;51). Interestingly *PTEN* mutations, like *EGFR* amplifications, are found almost exclusively in primary GBMs and there is a frequent association between the EGFR amplification and loss of 10q (where the *PTEN* suppressor gene is located) in GBMs (62). In fact 10q losses are part of the primary GBM genetic signature (3). However there was no significant correlation between the presence of *EGFR* amplification and *PTEN* mutations (63). In any case there are some important clinical implications as the presence or absence of functional PTEN could influence the effectiveness of certain EGFR-targeted molecular therapies (see below).

STAT3 activation. STAT3 is a latent transcription factor found in the cytoplasm of cells. It is activated by tyrosine phosphorylation, leading to dimerization, nuclear translocation, DNA

binding, and gene activation. STAT3 is transiently activated in normal cells but constitutively activated in a wide variety of hematologic and epithelial primary tumors, and also in astrocytomas (64). STAT3 tyrosine phosphorylation is induced by stimulated EGFR although it can also be induced by stimulation of other upstream receptor and/or no receptor kinases including PDGFR, Src and JAK2 (Janus kinase 2) (65). Interestingly, a recent work by Lee and co-workers, has indicated that in GBM STAT3 is further activated by enhancer of Zeste homolog 2 (EZH2)-mediated methylation. This alternative mechanism is induced by AKT phosphorylation of EZH2 suggesting RTK-independent activation of STAT3 in PI3K pathway- mutated GBM (66).

Similarly to what it happens to MAPK activation, there is no reports of STAT3 gain-of-function mutations in GBM; rather, the activation of STAT-3 is thought to be a consequence of either deregulation of upstream kinases or loss of endogenous inhibitors (67;68). It has been associated with the control of cell cycle progression, apoptosis and immunosuppression in GBM (67;69) and many studies indicate the anti-neoplastic potential of STAT3 inhibitors in GBMs (70). Regarding EGFR-mediated activation some groups have reported that STAT3 constitutive activation coexists with EGFR expression in almost 30% of high-grade gliomas and that targeting STAT3/JAK2 sensitizes these tumors to anti-EGFR agents (68). However other authors have reported that STAT-3 phosphorylation correlates only with the presence of EGFRvIII (57) suggesting that is specifically activated in the presence of such mutation. These findings could be related to the increased gliomagenesis potential of this mutant receptor form (see below).

PLCγ-PKC signaling. The protein kinase C (PKC) pathway also plays an important role in mediating the effects of activated growth factor receptors including EGFR. Phospho-lipase Cγ (PLC-γ) is recruited and phosphorylated by EGFR. Activated PLCγ in turn interacts with the plasma membrane where it cleaves PIP_2 to inositol triphosphate (IP_3) and diacyglycerol (DAG). IP_3 can bind its receptors on the endoplasmic reticulum (ER) to induce calcium (Ca^{2+}) influx into the cell. Ca^{2+} and DAG can activate the serine/threonine kinase activity of PKC that in turn phosphorylates a plethora of substrates regulating proliferation, apoptosis, cell survival and cell migration (71). In GBM, survival in patients with tumors expressing PKC or PLCγ was significantly shorter (49). A novel study has indicated that PLCγ signaling, in response to GBM EGFR activation, induces IKKβ and leads to NFκB translocation (72). As we just mentioned NFκB activity has been linked to the suppression of apoptotic signals. In fact this transcription factor cooperates with EGFR in breast (73) and lung cancer (74) and aberrant constitutive activation of NFκB has been observed in glioblastomas (75). Bredel and co-workers have recently demonstrated that *NFκI1A*, the gene that codes for the NFκB inhibitor (IκBα), is often deleted in these tumors. They have further showed that deletion of IκBα has a similar effect to that of EGFR amplification in the pathogenesis of GBM and is associated with comparatively short survival (76). These results suggest that activation of NFκB is another fundamental pathway for glioma progression and that it can be achieved either by genetic deletion of its inhibitor, or by *EGFR* amplification.

Apart from its role in apoptosis suppression, nuclear NFκB also cooperates with other transcription factors like Hypoxia-inducible factor 1α (HIF1α). This interaction can lead to an overexpression of pyruvate kinase M2 (PKM2) (72). PKM2 catalyzes the last step within glycolysis, the dephosphorylation of phosphoenolpyruvate to pyruvate, and is responsible for

net ATP production and the accumulation of lactate within the glycolytic sequence (77). Tumor cells have elevated rates of glucose uptake and higher lactate production, even in the presence of oxygen. This phenomenon, known as aerobic glycolysis or the Warburg effect, supports tumor cell growth. Accordingly PKM2 expression is increased and facilitates lactate production in cancer cells (78;79) and a higher expression of PKM2 has been shown in GBM compared to lower grade gliomas and normal tissue (80). In fact Yang and co-workers have postulated PKM2 upregulation as the key step for EGFR-promoted glycolysis, demonstrating a good correlation between PKM2 expression and EGFR and IKKβ activity in GBM samples (72).

PKM2 is also located in the nucleus, in fact it has been shown to function as a co-factor for HIF-1α, facilitating the transcription of hypoxia responsive genes and further promoting glucose uptake and lactate production (81). Therefore PLCγ-PKC signaling, through the activation of NFκB and the overexpression of PKM2, links EGFR activation to the regulation of glycolysis and the hypoxia response, contributing to the survival of the GBM cells in their harsh tumorigenic niche. Furthermore it has been shown that PKM2 is required for EGFR activation-induced β-catenin transactivation (82). These authors postulate that brain tumor development promoted by EGFR requires PKM2-modulated β-catenin transactivation, suggesting a crosstalk between EGFR and Wnt signaling in the regulation of glioma cell growth.

EGFR-Src interaction. Although the major tyrosine sites of the EGFR C-terminal domain seem to be auto-phosphorylated, some tyrosine residues are phosphorylated by other intracellular tyrosine kinases. For example, EGFR Y845 is phosphorylated by Src, also known as tyrosine-protein kinase CSK. Src and EGFR form an EGF–dependent heterocomplex *in vivo*; this interaction is mediated by Src's SH2 domain, which can directly bind to phospho Y891, Y920 and Y1101 sites (83;84). The non-receptor tyrosine kinase Src was one of the first oncogenes identified, and the SFKs collectively regulates a variety of cellular functions in many cancer types including proliferation, invasion, motility, survival, differentiation, and angiogenesis (85). Co-overexpression of EGFR and Src frequently occurs in human tumors and is linked to enhanced tumor growth. Src is capable of potentiating receptor-mediated tumorigenesis, causing synergistic increases in EGF-induced DNA synthesis, soft agar colony growth, and tumor formation in nude mice (86-88). Recent work indicates that SFKs are frequently coactivated with EGFR in GBM cell lines and patients (89) and it has been shown that dasatinib (a SFK inhibitor) enhances the efficacy of EGFR-targeted therapies in these tumors (36).Moreover it has been proposed that Src-dependent EGFR activation is induced by IR in glioma cells (90). All the above provide a rationale for combined anti-EGFR and anti-SFK therapies

One of the proposed binding targets for EGFR phospho-Y845 is STAT5b (a transcription factor known to be overexpressed in several tumors) that could mediate the synergism between Src and EGFR (91). In GBM STAT5 seems to be preferentially activated by EGFRvIII (92) and the STAT5b isoform has been shown to be overexpressed compared to normal tissue or lower grade gliomas. Moreover it has been linked to the control of GBM cell growth, cell cycle progression, invasion and migration through regulation of gene expression (93).

Another pathway that appears to be regulated through Y845 following EGF stimulation is that mediated by the cytochrome-c oxidase subunit II (CoxII)-related trafficking of EGFR to

mitochondria, the central organelles that produce energy and initiate apoptosis (94). The catalytic activity of EGFR and Src, as well as endocytosis and a mitochondrial localization signal are required for these events. Rapamycin, apoptosis inducers, and EGFR inhibition can further enhance EGFR mitochondrial transport (95;96). Once in the mitochondria CoxII can be phosphorylated by both EGFR and c-Src, reducing Cox activity and cellular ATP. These findings suggests EGFR plays a novel role in modulating mitochondrial function via its association with, and modification of CoxII and contributing to regulate cell survival (94;97). Although the relevance of the EGFR-CoxII interaction in GBM remains to be determined, the Src induced localization of EGFRvIII is stimulated in conditions of low glucose and this mutant EGFR reduced glucose dependency by stimulating mitochondrial oxidative metabolism (98). Interestingly the amount of mitochondrial EGFR seems to be fine-tuned by the balance between autophagy and apoptosis, inhibition of the first or induction of the second provokes an accumulation of EGFR in this organelle as a pro-survival mechanism (96). The study by Cao and coworkers indicated that both wild type and vIII EGFR can translocate to the mitochondria when induced by apoptosis-inducing agents and EGFR kinase inhibitors, and that tumor cells with accumulated mitochondrial EGFR are resistant to apoptosis induced by these agents (95). Taken together these studies suggest that tumor cells reprogram their intracellular trafficking of EGFR/EGFRvIII by increasing its mitochondrial accumulation, as a mechanism for escape from therapy- and stress-induced apoptosis and growth suppression. Moreover they suggest that targeting mitochondrial translocation could synergize with EGFR inhibitors and classical therapies.

Nuclear EGFR signaling. Throughout the previous paragraphs we have mentioned that most of the EGFR-activated signaling pathways end up in the nuclear translocation of secondary messengers and in the modulation of several transcription factors activity. However EGFR itself has been often detected in the nuclei of cancer cells, primary tumor specimens, and other highly proliferative tissues (99). Increased nuclear EGFR localization correlates with poor clinical outcome in several types of cancer (100). Although this analysis has not been performed in gliomas both, EGFRwt and EGFRvIII, have been detected in the nucleus of normal glial cells and primary GBM specimens, where they cooperate with STAT3 function (101;102).

Recent reports have characterized a novel nuclear localization sequence (NLS) comprising amino acids 645 to 657, adjacent to the transmembrane domain in EGFR and its family members (103). This 13 amino acids sequence has a dual role: it mediates EGFR allosteric conformational change and dimer stabilization, which are indispensable for the receptor activation, but it also allows nuclear translocation via binding to importin β (104). Furthermore cumulative evidence indicates that EGFR internalization serves to transport the receptor from the cell surface to different compartments within cells, like the mitochondria and the nucleus. Whereas further studies are required to determine if EGFR is integrated into the mitochondrial membrane through endosomal membrane fusion, it has been demonstrated that disruption of receptor internalization suppressed nuclear entry of EGFR (105). Once in the nucleus EGFR still functions as a tyrosine kinase, phosphorylating and stabilizing PCNA and thus enhancing the proliferative potential of cancer cells (106). This could explain the strong correlation between nuclear localization of EGFR and the highly proliferative status of tissues (99). It remains to be determined if there are other possible substrates for EGFR and/or EGFRvIII in the nucleus.

Nuclear EGFR and DNA damage regulation. EGFR overexpression has been implicated in radioresistance in a variety of human cancers, including GBM. Moreover it has been correlated with poor radiographic response to radiation therapy in some patients with this tumor (9;107). EGFR itself can be activated by radiation in a ligand-independent way, promoting cancer cells survival and proliferation. Furthermore, EGFR and EGFRvIII have been linked, by several authors, to the repair of double-strand breaks (DSB) (the most lethal DNA lesions induced by ionizing radiation (9;108). In fact the use of EGFR inhibitors in GBM cell lines and intracranial xenografts caused tumor regression when combined with radiotherapy (109;110). It appears that both PI3K and ERK pathways mediate the signals downstream of the receptor in order to activate DNA-dependent serine/threonine protein kinase (DNA-PK), which is required for non-homologous end joining (NHEJ) of DSBs (111;112). Moreover, the disruption of PI3K/AKT pathway signaling by small-molecule inhibitors blocks DSB repair in GBM, whereas PTEN loss promotes it, resulting in radioresistance (113). More recent discoveries indicate that nuclear EGFR can influence DNA repair directly via physical interaction with DNA-PK. The EGFR antibody, cetuximab, decreased nuclear DNA-PK protein and kinase activity by reducing its physical interaction with the receptor (114). Moreover cetuximab blocks nuclear shuttling of EGFR and prevents phosphorylation of DNA-PK and DSB repair (115;116). Altogether these suggest that DNA-PK inhibitors and/or EGFR inhibitors may be an effective therapeutic strategy for radiosensitizing GBM tumors.

EGFR expression in gliomas

The frequent amplification of the *EGFR* gene in GBM was initially reported in 1985 by Libermann and coworkers (117) and it has been confirmed in many subsequent studies. It has been estimated that 30 to 40% of GBM exhibit *EGFR* gene amplification and nearly 50% of them overexpress the receptor (1;6) (7). Although it is not well understood, high levels of *EGFR* mRNA are also present in less malignant astrocytomas and oligodendrogliomas without the underlying gene amplification (118). These observations underlie the relevant function of EGFR in glial cells and suggest that other oncogenic events may lead to increased transcription of this gene.

Amplification of *EGFR* has been reported in only 3% of anaplastic (grade III) astrocytomas (119) and is infrequent in secondary GBMs (only 8%) whereas 60% of primary GBMs show EGFR overexpression and 40% of them contain *EGFR* amplifications. In consonance with this *EGFR*-amplified GBM are rare in patients younger than age 35. With increasing patient age, *EGFR* amplification becomes more common and the median age of GBM patients with this alteration is 62 years (3). Pediatric GBMs are rare tumors and show genetic differences compared with adult GBMs, including a much lower prevalence of *EGFR* amplification (0%-5%) and EGFR protein overexpression (25%) (120). From the histopathological point of view EGFR gene amplification is relatively common in small cell GBMs (69%) but rare in gliosarcomas (0%) and giant cell GBMs (6%) (107).

There have also been several correlation studies between the *EGFR* status and changes in other common GBM pathways. In general there is a tendency for mutual exclusion between *EGFR* alterations and mutations in the tumor suppressor *p53* (120-124). Indeed they are considered as hallmarks of primary and secondary GBM respectively (3). On the other hand the RB1 pathway seems to be important in both primary and secondary GBM. However,

homozygous deletions of the *INK4A-ARF* locus, which encodes tow gene products (*p16INK4A* and *p19ARF*) involved in cell-cycle arrest and apoptosis, are more frequent in primary than in secondary tumors (3). Moreover there is a frequent association of *INK4A/ARF* loss of function and activation of *EGFR* in GBM (125), raising the possibility that critical functional interactions between these mutations are necessary for cellular transformation as it was lately corroborated in several *in vivo* mouse models (see below).

From the neuropathology point of view, identification of *EGFR* amplification or the presence of EGFRvIII constitute strong evidence that the tumor is a GBM, or at least that it should be treated like one, even in the absence of necrosis and microvascular proliferation in the biopsy (126). On the other hands, in patients with *EGFR* amplification, multivariate analysis revealed that EGFRvIII overexpression was an independent, significant, poor prognostic factor for overall survival (127). However, although nobody doubts about the diagnostic value of *EGFR* analysis in GBM, there are some discrepancies regarding the prognostic value of *EGFR* amplification/overexpression, especially when patients of all ages are analyzed together (16). In fact *EGFR* amplification has been associated with a worse prognosis in younger patients but with a better prognosis among older patients (124;127-129). Moreover, among the younger patients, the *EGFR* amplification predicted worse prognosis only in those with tumors without p53 mutations, suggesting that the oncogenic potential of the receptor could be overcome by alterations in the tumor-suppressor pathway (130). *EGFR* amplification is also present (26%) in long term GBM survivors (patients surviving longer than 3 years) (131) suggesting that this oncogenic pathway is not much more aggressive than other GBM related alterations.

EGFR and GBM molecular profiling

During the last years, the availability of high-throughput profiling techniques has allowed the identification of molecular subclasses of an otherwise apparently uniform disease. As a proof of principle specific expression profiles were found to distinguish robustly primary from secondary GBM (132;133) demonstrating that they are two different entities. More recently The Cancer Genome Atlas (TCGA), using unsupervised clustering of global transcriptional data, designated four GBM subclasses: proneural, neural, classical and mesenchymal (5). An earlier transcriptional study analyzing a set of grade III and IV gliomas, established three molecular variants of malignant glioma with at least two of them (also name proneural and mesenchymal) being very similar to those of the TCGA analysis (4;134). Interestingly the proneural subgroup has a better prognosis and this gene expression signature is enriched in anaplastic astroctyomas and also in tumors with oligodendroglioma histology (135). In accordance with these observations proneural tumors are more common in younger patients. In fact the beneficial effect of younger age in patients diagnosed with GBM is entirely due to the higher proportion of proneural tumors among them (136). Moreover, the gene signatures of the different subgroups correlated best with different cell lineages. Neural tumors correlated best with mature neurons, proneural tumors with oligodendrocytes, and mesenchymal and classical tumors with astrocytes (5), suggesting that the different GBM subtypes could have different cells of origin. In fact, when GBM cells are grown in mouse

xenografts or in cell culture they retain their molecular differences, reinforcing the notion that there are different GBM tumor-initiating-cells (4;5). However it is important to keep in mind that the GBM molecular subclasses are not homogenous and that many tumors may be composed of different subpopulations.

The different GBM groups correlate with defined genomic abnormalities. More specifically, proneural tumors have been strongly associated with alterations in platelet-derived growth factor receptor alpha (*PDGFRA*) and *IDH1,2,* and mesenchymal tumors with mutations in *NF1* (5). Regarding *EGFR*, the TCGA analysis indicates that gene amplification, and in particular the presence of the vIII isoform, are enriched in the classical GBMs, although it is also present in the other subtypes (5). On the other hand Phillips and coworkers suggested that chromosome 7 (where *EGFR* gene is located) amplifications are more frequent in the mesenchymal subtype (4). Apart from the genomic studies, a proteomic analysis further supports three basic subdivisions in malignant glioma characterized by NF1 expression, upregulated PDGF signaling, or upregulated EGF signaling, highly reminiscent of the genomic abnormalities enriched in mesenchymal, proneural and classical GBMs respectively (137). Regarding the classification of GBM cells, grown *in vitro* as primary cultures, most groups define only two subtypes, characterized by differential expression of markers like CD133 and Olig2 in one group, and CD44 in the other. They have been so called as proneural and mesenchymal GBM cells based on their expression profiles (138-141) although *EGFR* amplification have been described in both groups (139). Another study, however, have suggested the existence of three different subgroups of GBM cells, with *EGFR* being amplified and expressed in neurospheres expressing the signature of the classical subtype and *MET* (encoding a protein known as hepatocyte growth factor receptor, HGFR) being present in the mesenchymal and proneural subtypes (142). Likewise it has been suggested that the gene expression profiles of glioma subtypes overexpressing *EGFR* are distinct from the rest (143) suggesting that *EGFR* alterations drive a specific tumor developmental program and that EGFR addicted GBM might behave differently from other aggressive gliomas.

EGFR and GBM tumor initiation: mouse models of GBM related to EGFR

The extraordinary presence of *EGFR* alterations in primary GBMs suggest that this receptor participates not only in tumor growth but also during tumor initiation. In fact numerous investigations carried out using animal models, including transgenic mice, have indicated that the enhanced expression of EGFRwt and especially truncated EGFRvIII, in neural stem cells (NSCs) or more committed neuronal or glial precursor cells, can cooperate with other genetic alterations to induce primary brain cancer initiation and progression (Table 1). Moreover these mouse models provide insight into the participation of EGFR in tumor maintenance and also in the resistance to therapeutic intervention, either to unspecific cytotoxic agents or to EGFR tyrosine kinase inhibitors.

In accordance with the strong correlation between *EGFR* gene alterations and loss of the *INK4A-ARF* locus (125), most of the glioma models based on overexpression of wild type or vIII EGFR have been obtained in cells that are deficient for this tumor suppressor. The first model

described made use of avian retroviral vectors to transfer EGFRvIII into mice expressing *tv-a*, a gene encoding the retrovirus receptor, TVA, under the control of brain cell type-specific promoters, in an *Inka-Arf* null background (144). Interestingly the astrocytic lesions observed where much more frequent when the nestin promoter (progenitor-specific) was used than when the astrocyte-specific glial fibrillary acidic protein (GFAP)-tv-a mice were injected. In contrast EGFRvIII appeared incapable of generating gliomas in a p53-deficient background, unless CDK4 was also overproduced (145) suggesting an explanation for the mutual exclusivity of *EGFR* and *P53* mutations found in GBM (see above). A different approach was used by Bachoo and coworkers, as they used retroviral vectors to overexpress EGFR *in vitro,* in well-defined astrocyte or NSCs cultures from *Ink4a-Arf* deficient mice. Those cells were then reintroduced into the brains of SCID mice. Their results showed that both compartments are equally permissive for the generation of high-grade gliomas (146). With a similar approach, *in vitro* EGFRvIII expression in *PTEN* deficient NSCs was able to synergistically induce chromosomal instability and form astrocytic tumors (147). These data suggest that deregulation of specific genetic pathways, rather than the cell-of-origin, dictates the emergence and phenotype of high-grade gliomas. However, the analysis of a transgenic mouse model that express the v-*erbB* oncogene under the control of *S100b* promoter, demonstrated the appearance of low grade oligodendrogliomas in 20% of the mice. These lesions were more aggressive and the penetrance was higher in the context of *p53* or *Ink4-Arf* heterozygous mice (148). *S100b* is expressed by oligodendroglia and astrocytes during early brain development although it is also present in NSCs so it is difficult to conclude which ones where the cell of origin of the tumors observed (149). However, the differences with the previous models suggest that oligodendrocytes are more readily transformed by v-erbB, at least during early neural development.

Other groups have demonstrated that EGFRvIII overexpression can cooperate also with oncogenic Ras mutations. In this case the Ras astrocytoma-prone model (RasB8 mice: GFAP-V12Ha-ras transgenic mice) was used (150). Ding and coworkers made double transgenics to express EGFRvIII and mutated Ras in the same cells and they observed an increase in the penetrance of the tumors (compared to the single Ras transgenic mice). However they also reported a change on the phenotype as overexpression of EGFRvIII leaded to the appearance of oligodendrogial and mixed oligoastrocytoma (151). Interestingly the same authors demonstrated that GFAP-EGFRvIII astrocytes, forced to express mutated Ras *in vitro*, generated oligodendroglioma-like tumors when inoculated back into immunodeficient brains. By contrast injection of adenovirus expressing EGFRvIII in adult RasB8 brains was able to induce low grade and high grade astrocytomas with a high penetrance (152). These results, together with the studies in the *Ink4-Arf*-/- background confirm that expression of EGFRvIII is not sufficient to initiate gliomagenesis although it cooperates with other genetic alterations to induce glioma formation. They also suggest that the same EGFR mutation can generate tumors with astrocytic or oligodendrocytic phenotype, depending on the cell type and the developmental stage in which the alteration takes place. However it is still not known if EGFRvIII activates different signaling pathways in the germline and in the somatic context that could explain the changes in the phenotypic outcomes.

An important corollary from these mouse models is that EGFRwt cannot substitute for EGFRvIII in driving infiltrative glioma formation in genetically engineered mice (151) or in *Ink4-*

Arf-/- cells (145;146). One possible explanation for these phenomena would be that a sustained EGFR signaling is necessary for glial transformation. The mitogenic effect of EGFRvIII have been explained by a low but constitutively active kinase activity, amplified by failure to attenuate signaling by receptor down-regulation (153). However, overexpression of self-activating EGFRwt levels might not be sufficient to induce cell transformation due to its constant lysosomal targeting. The results of Zhu and coworkers are in agreement with this hypothesis. They used a conditional transgenic model based on somatic induction of vIII or EGFRwt in adult animals. In these models expression of EGFR is triggered by stereotactic injection of an adenovirus expressing Cre recombinase. In consonance with the results of the previous groups they reported that expression of EGFRvIII, concomitant with loss of the *cdkn2a* and/or *PTEN* locus, promotes the formation of aggressive gliomas (33). Interestingly, they also showed that overexpression of EGFRwt, to levels comparable to those observed in human GBMS, is very inefficient at forming tumors under the same conditions (33). Later on, the same group used bicistronic lentiviral vectors designed to express TGFα and Cre recombinase so that they can induce EGFR expression in *Ink4-Arf*-/- and/or *PTEN* deficient mice. They demonstrated that somatic, ligand-mediated activation of EGFR was necessary for gliomagenesis (154;155) further supporting that persistent EGFR signaling is a necessary oncogenic event. These results are in agreement with the clinical observation of common overexpression of EGFR ligands in receptor-amplified GBM (see above).

Although many of these mouse models demonstrate the greater biological activity of the truncated vIII variant of EGFR, in patients this mutation occurs almost exclusively together with *EGFR* amplifications, suggesting a crosstalk between the mutant and the wild type receptors in human GBM cells. This cooperation could be explain by a cell-autonomous manner as the vIII isoform can be a substrate for EGFRwt and this phosphorylation triggers nuclear entry of EGFRvIII and STAT3 activation (156). However autocrine and/or paracrine crosstalks have also been proposed. It has been shown that EGFRvIII induces expression of EGFR ligands (HB-EGF) whereas EGFRwt activates the mutant isoform by facilitating EGFRvIII dimerization (157), suggesting a feed-forward loop that regulates the oncogenic action of the receptors. More recently it has been reported that the EGFRvIII-expressing cells release cytokines (like interleukin 6 (IL6) and leukemia inhibitory factor (LIF)) that activate neighboring EGFRwt-expressing cells, favoring the formation of heterogeneous gliomas in mice (158). These non-cell autonomous effects could explain the coexistence of the mutation with gene amplification in the same tumor but not always in the same cells.

Animal models can also provide opportunities to determine whether EGFR activation generates a specific GBM subclass. Although it has been described the formation of oligodendroglial histological tumors in transgenic models expressing vErbB, the rest of the EGFR-related mouse studies reported the appearance of astrocytic tumors (Table 2), which could be related with the absence of *EGFR* alterations in oligodendrogliomas. In fact Jun and coworkers have shown that the TGFα-EGFRwt; *Ink4-Arf*-/- tumors resemble molecularly the classical GBM subtype (155), the one that presents a higher frequency of *EGFR* alterations (5). However their results also indicate that the same combination of mutations generates mesenchymal-like GBM in the absence of PTEN expression (155). It remains to be confirmed if a similar combination of mutations occurs in human tumors classified in this subgroup.

Table 1. GBM mouse modeling linked to EGFR

Driving mutations	Method for EGFR overexpression	Target cells	Observations	Reference
EGFRvIII *Ink4-Arf -/-*	RCAS viral vectors	Nestin + GFAP +	Astrocytomas Much higher penetrance in Nestin + cells	(145)
EGFRvIII Cdk4	RCAS viral vectors	Nestin +	Astrocytomas Higher penetrance in the absence of p53	(145)
EGFRvIII *Ink4-Arf -/-*	In vitro transduction	*Ink4-Arf -/-* Ast. and NSCs	High grade Astrocytomas and mixed tumors	(146)
EGFRvIII *PTEN* loss	In vitro transduction	*PTEN -/-* NSCs	Astrocytomas	(147)
v-erbB	Transgenic mice	S100b +	Low Grade Oligodendrogliomas	(148)
v-erbB; *Ink4-Arf -/-*	Transgenic mice	S100b +	High grade Oligodendrogliomas	(148)
v-erbB; *p53-/-*	Transgenic mice	S100b +	High grade Oligodendrogliomas	(33)
EGFRvIII; V12Ha-Ras	Transgenic mice	GFAP + in RasB8 mice	Oligodendrogliomas	(151)
EGFRvIII; V12Ha-Ras	Transgenic mice *in vitro* transduced Ras	GFAP +	Oligodendrogliomas	(151)
EGFRvIII; V12Ha-Ras	Adenoviral *in vivo* injections	RasB8 brains (GFAP +)	Low grade and High grade Astroctyomas	(152)
EGFRwt *Ink4-Arf -/-* PTEN loss	Cond. Exp. (AdCre) *in vivo* injections	*Ink4-Arf -/-* *PTEN*^{2Lox} brains	Highly aggressive gliomas Very low penetrance	(33)
EGFRwt +EGFRvIII *Ink4-Arf -/-* *PTEN* loss	Cond. Exp. (AdCre) *in vivo* injections	*Ink4-Arf -/-* *PTEN*^{2Lox} brains	Higher penetrance than EGFRwt alone	(33)
EGFRviii *Ink4-Arf -/-* PTEN loss	Cond. Exp. (AdCre) *in vivo* injections	*Ink4-Arf -/-* *PTEN*^{2Lox} brains	Lower latency than in the presence of EGFRwt	(33)
EGFRwt-TGFα *Ink4-Arf-/-*	Cond. Exp. (lenti-TGFα-Cre) *in vivo* injections	*Ink4-Arf -/-* brains	High grade astroctyomas	(154)
EGFRwt-TGFα *PTEN* loss	Cond. Exp. (lenti-TGFα-Cre) *in vivo* injections	*PTEN*^{2Lox} brains	Reduced penetrance and longer latency than in the *Ink4-Arf-/-* mice	(155)
EGFRwt-TGFα *Ink4-Arf-/-* *PTEN* loss	Cond. Exp. (lenti-TGFα-Cre) *in vivo* injections	*Ink4-Arf -/-* *PTEN*^{2Lox} brains	Reduced penetrance and longer latency than in *Ink4-Arf-/-* or *PTEN* loss mice	(155)
EGFRwt + EGFRvIII *Ink4-Arf -/-*	In vitro transduction	*Ink4-Arf -/-* Ast.	EGFRwt and EGFRvIII expressed by different cells	(158)

RCAS: Replication-Competent ALV Splice acceptor

Ast.: Astrocytes

NSCs: Neural Stem Cells

RasB8 mice: glioma-prone mice expressing Ha-Ras (V12Ha-Ras) in GFAP positive (+) cells

Con. Exp.: Conditional EGFR Expression using the lox-stop-lox EGFR^{WT} transgenic mouse strain

AdCre: Adenovirus transducing Cre recombinase

PTEN^{2LOX}: Conditional PTEN loss

lenti-TGFα-Cre: Lentiviral bicistronic vector transducing TGFα and Cre recombinase

Targeting EGFR in GBMs

After the discovery that EGFR was implicated in the development of a variety of epithelial cancers the receptor began to be considered as an interesting therapeutic target. Several anti-EGFR based therapeutic strategies have been assessed as monotherapy, or in combination with radio and conventional chemotherapy, in pre-clinical and clinical trials. Here we review the most promising results and ongoing clinical trials in GBM patients (Table 2).

Antibodies against EGFR. Anti-EGFR monoclonal antibodies (mAbs) have been tested in cancer, in an attempt to block the binding of EGF to its receptor and therefore to avoid the activation of the downstream signal transduction pathways. The mAb225 (C225), also known as cetuximab (Erbitux® [Bristol-Myers Squibb, ImClone Systems]) is currently in phase I/II to study the efficacy of its combination with radiotherapy and TMZ to treat patients with primary GBM, and also as a second line treatment in combination with bevacizumab (anti VEGF receptor, VEGFR, antibody) and irinotecan (159;160)) (reviewed in (161)). More recently, a randomized phase III study using the humanized mAb nimotuzumab (hR3, Theraloc®; YM BioSciences Inc.) in first line GBM treatment combined with chemo and radiotherapy, has shown some survival benefit (162). Furthermore several antibodies targeting specifically the truncated receptor have been developed. mAb806, for example, binds to the short cysteine loop of the extracellular domain that is always exposed in EGFR vIII and attenuates receptor autophosphorylation. Due to mAb806 tolerance, great biodistribution and specificity for its target in GBM patients, several clinical trials with a humanized version of mAb806 (ABT-806; Abbott) are being currently performed (163;164).

The strongest reason for the use of EGFR directed antibodies in GBM patients is that they provoke fewer side effects than the traditional chemotherapy treatments. However it is still not known if their capacity to go through the BBB will be enough to improve the results obtained with small molecules and the cost-effectiveness ratio is still very high.

Vaccination against EGFR vIII. Immunotherapy is also an attractive alternative to cytotoxic strategies as they could eliminate tumor cells with reduced toxicity. The current strategies in GBM are focused on targeting the vIII-truncated form of EGFR. The generation of a new amino acid sequence (PEPvIII) with immunogenic capacity, due to the loss of exons 2-7 in the truncated EGFRvIII and the fusion of two distant regions of the wild type molecule, makes it an appropriate target for peptide-based vaccination (165). Rindopepimut (CDX-110, Celldex) is an experimental vaccine containing the amino acid sequence PEP-vIII linked to the carrier protein keyhole limpet hemocyanin (KLH), to generate both humoral and cellular immune responses. This approach has shown ability to eliminate the EGFRvIII-expressing cells as a high proportion of the relapsing tumors after rindopepimut treatment show no EGFR vIII reactivity. Moreover it has been reported that the combination of rindopepimut with TMZ improves both progression free survival (14.2 months vs. 7.3 months) and median survival (26 months vs. 15. 2 months) in GBM patients (166). However, one of the main caveats of this approach is the intratumoral heterogeneity of GBMs as the antigen is being expressed only by a subgroup of tumor cells. Therefore it is still not known if this immunotherapy will induce long-term reduction of the tumors.

Small-molecule tyrosine kinase inhibitors (TKIs). The development of small-molecule TKIs was simultaneous to the generation and improvement of the anti-EGFR mAbs. However they are nowadays the most advanced EGFR-based therapies in the clinic. The best-studied TKIs are quinazoline-derived synthetic molecules with low molecular weight, which are able to block the magnesium-ATP- binding pocket of the intracellular TK domain. This union prevents the activation of the kinase domain by ligand-induced auto-phosphorylation and downstream activation of survival signaling pathways (167).

The first EGFR-specific TKIs used in the clinic for the treatment of newly diagnosed and recurrent gliomas were gefitinib (Iressa®, ZD1839; AstraZeneca), erlotinib (Tarceva®, OSI774; Genentech), which are EGFR/HER1 specific, and lapatinib (Tykerb/Tyverb; GSK), with ability to block both EGFR/HER1 and HER2. However, in spite of the promising pre-clinical results, obtained both *in vitro* and *in vivo* with these first generation TKIs, they have not accomplished the expectatives. In two recent phase II clinical trials for GBM therapy, erlotinib was well tolerated, but showed no clinically meaningful results, only a modest effect over placebo (168;169). Regarding gefitinib, a phase II clinical trial showed that it was well tolerated and displayed anti-tumor activity, but the median overall survival time in GBM patients was only 38.4 weeks from treatment initiation, which supposed a very modest clinical benefit (10).

These findings led to the design of second generation TKIs, which are able to bind irreversibly the ATP binding site of various HER receptors (pan-HER inhibitors) such as afatinib (Gilotrif®, Boehringer Ingelheim), which binds EGFR/HER1 and HER2, and dacomitinib (PF-0299804, Pfizer) that bind to EGFR/HER1, HER2 and HER4, among other TKIs. Most of these second generation TKIs have proven some efficacy in other tumors and they are currently in clinical testing with GBM recurrent patients (Table 2). However while we wait for the results of these next generation TKIs we need to reconsider the possible explanations for the lack of therapeutic response observed until now with EGFR-directed strategies in GBM patients.

	Agent	Brand name	Company	Target	Class	Selected references/ Trial identifier
Monoclonal antibodies	Cetuximab (C225)	Erbitux®	ImClone Systems Inc.	EGFR/HER1	Mouse-human chimeric antibody	(159;160)
	Nimotuzumab (h-R3)	TheraCIM®	YM Biosciences	EGFR/HER1	Human antibody	(162)
	Pantitumumab	Vectibix®	Amgen	EGFR/HER1	Human antibody	NCT01017653
	^{125}I-MAb 425		Fox Chase Cancer Center	EGFR	Radiolabeled murine antibody	(170)
	mAb 806	ABT-806	Abbott	EGFR vIII	Human antibody	(164)
Small-molecule tyrosine kinase inhibitors	Gefitinib (ZD1839)	Iressa®	Astra Zeneca Pharmaceuticals	EGFR/HER1	Aniliquinazoline-based reversible inhibitor	(10)
	Erlotinib (OSI-774)	Tacerva®	Genentech Inc.	EGFR/HER1	Aniliquinazoline-based reversible inhibitor	(168;169)
	Lapatinib (GW572016)	Tykerb®	GlaxoSmithKline	EGFR/HER1, HER2	Thiazolylquinazoline based reversible inhibitor	(171)
	Afatinib	Gilotrif®	Boehringer Ingelheim	EGFR/HER1, HER2, HER4	Anilinoquinazoline based irreversible inhibitor	NCT00977431
	Dacomitinib (PF-00299804)		Pfizer	EGFR/HER1, HER2, HER4	Anilinoquinazoline based irreversible inhibitor	NCT01520870
	Vandetanib (ZD6474)	Zactima®	AstraZeneca Pharmaceuticals	EGFR/HER1, VEGFR	Aniliquinazoline-based inhibitor	(172)
	Pelitinib (EKB-569)		Wyeth Pharmaceuticals	EGFR/HER1	Cyanoquinoline-based irreversible inhibitor	
Vaccines	Rindopepimut (CDX-110)		Celldex Therapeutics	EGFRvIII	Peptide vaccination	ACT VI

Conclusions from TKI studies in GBM: synergistic approaches and predictive markers

There are several explanations for the EGFR-targeted therapy failure in GBM patients. Deficient tumor drug penetration and TKIs systemic availability reduction by enzyme inducing antiepileptic drugs, which are used in most GBM clinical trials, could be some of the reasons behind the lack of success of these compounds (8;161;167). However, beyond the drug delivery limitations, some GBM molecular features could be responsible for the limited benefit provided by these therapeutic agents.

EGFR vIII mutation. It has been proposed that EGFR vIII-positive cells are resistant to gefitinib because they need greater amounts of the drug and a longer exposition to it to reduce EGFR downstream signaling. Moreover tumors bearing EGFRvIII have a worse response to cetuximab (173). It has been argued that the strong activation of the PI3K/AKT signaling pathway by EGFR vIII cannot be reduced by gefitinib treatment, leading to the persistence of tumor cell proliferation and survival signals in the presence of this TKI (167;174;175). By contrast Mellinghoff and coworkers described that expression of EGFRvIII, in a wild type PTEN context, is associated with GBM responsiveness to EGFR kinase inhibitors (176). However these findings could not be confirmed in subsequent trials, including a randomized phase II trial (168). The results of the new upcoming trials with next generation TKIs will help to understand the relevance of the presence of the vIII isoform as a predictive marker.

PTEN deletion and phospho-AKT levels. AKT basal activation levels (due to activation by other RTKs or by PTEN deletion) seem to be one of the causes that could explain EGFR inhibitors failure in several types of cancer. It was reported, for example, that PTEN restoration in a PTEN deficient line augments the response to EGFR TKIs, through the induction of higher levels of apoptosis (177). In GBMs it was proposed that patients carrying wild type PTEN tumors or low levels of phosphorylated PKB/AKT would have a better outcome in response to anti-EGFR treatments (12;176). PTEN function in tumors can be altered by post-traductional modifications such as oxidation, phosphorylation, acetylation and ubiquitination, leading to malignant transformation (178). It has been also shown that tyrosine phosphorylation of PTEN by SFKs and fibroblast growth factor receptor (FGFR) can modulate its function. In particular, PTEN phosphorylation at Y240 by FGFR is linked to EGFR-TKI resistance and reduced survival in GBM patients (179). These results suggest that checking PTEN status (both at the genetic and at the protein level) might be an important surrogate marker for EGFR directed clinical trials. Moreover they indicate that targeting PI3K-AKT pathway could add a beneficial effect to EGFR TKIs.

Different EGFR conformations in lung cancer and GBM. The EGFR mutations present in GBM and non-small cell lung cancer (NSCLC) have oncogenic transforming potential and promote high levels of basal phosphorylation of the receptor *in vitro*. However, the different location of the lung and brain tumor mutations has been associated with the diverse response of these cancers to EGFR inhibitors. Crystallography studies have indicated that EGFR TKIs bind to different configuration of the receptors. When coupled with gefitinb and erlotinib the receptor shows an active conformation, also known as “type I” conformation, which is related to mutations in the intracellular kinase domain and frequent in NSCLC such as the deletion of exons 19 and 21 and point mutations in the exons 18-21, which sensitize tumor cells to EGFR targeted therapies (27;28;180). In complex with lapatinib, by contrast, EGFR is in an inactive configuration, also

called “type II” conformation, which is typical of the ectodomain mutations found in GBM samples, including missense and in frame deletions such as the EGFR vIII variant. It is not surprising then that glioma cells carrying extracellular EGFR mutants were poorly inhibited by erlotinib, whereas type II inhibitors induced cell death in the same cells (180). Furthermore Barkovitz and coworkers confirmed that EGFRvIII releases erlotinib more rapidly than wild type or lung cancer mutants and the kinase-site occupancy was correlated directly with cell-cycle arrest (181). The tropism of the TKIs for a particular receptor conformational state could explain why some agents are more effective in lung cancer than in GBM patients and supports the use of Type II EGFR inhibitors for GBM, although they should have a better brain penetrance than lapatinib.

Redundancy of receptor tyrosine kinase (RTK) signals. Treatment of GBM cells *in vitro* with gefitinib resulted in dephosphorylation of the EGFR, but also of most of the pathway regulators that have been mentioned above (Figure 2). However, analysis of the *in vivo* effect in established GBM xenografts or in the tissue of treated patients demonstrated that gefitinib efficiently dephosphorylates its target without significant effect on pathway constituents (182). These data suggest that there are compensatory mechanisms *in vivo*, probably mediated by other RTKs that share parts of their signaling pathways and show redundant regulatory circuit (183). One RTK that is ubiquitously expressed in cancer cells is the insulin-like growth factor receptor (IGF-1R). There is a functional cross-stalk between EGFR and this receptor as IGF-1R-deficient cells are resistant to transformation by EGFR (184). IGF-1R has been linked to GBM resistance to gefitinib through increased signaling of PI3K/AKT and the ribosomal protein S6 kinase. In addition, the pharmacological inhibition of IGF-R1 results in the sensitization of tumor cells to EGFR-TKIs treatment (185). Another RTK that is particularly expressed in GBMs is PDGFR. Interestingly the results from two different groups indicate that there is genetic heterogeneity in aggressive gliomas, with *EGFR* and *PDGFR* being amplified and activated simultaneously in adjacent intermingled cells (186;187). These results could suggest that combination of different inhibitors could be more efficient than EGFR-TKIs alone. However some recent evidences suggest that this is not necessary the case, as the addition of sunitinib (with capacity to inhibit several RTK including PDGFR and VEGFR) to gefitinib only improved the anti-GBM efficacy *in vitro,* but not in xenograft models (188). However Akhavan and coworkers have reported recently that inhibition of EGFR signaling de-represses the transcription of *PDGFRβ,* and that combined inhibition of both receptors potently suppresses tumor growth *in vivo* (189). Therefore there is still space for a synergistic approach targeting both signaling pathways.

Crosstalk EGFR-MET. The *MET* RTK is amplified in 5% of GBM although it is overexpressed in 30% of these tumors, being a bad prognosis factor (190). Moreover, MET is activated in GBM cells with increased levels of EGFR/EGFRvIII (183; 191). In fact there are evidences of autocrine and paracrine cross-talks between both signaling pathways (192). In line with these results resistance to EGFR inhibition can be overcome by using MET small molecule inhibitors (183;191) or neutralizing antibodies to hepatocyte growth factor (HGF), the MET ligand (192;193). Jun and coworkers have demonstrated that treatment of *EGFR* amplified mouse GBM cells with EGFR TKIs induces a cytostatic response, characterized, among other changes, by an increase in *MET* expression. Moreover they confirmed that pharmacological inhibition of MET overcomes the

resistance to EGFR inhibition by inducing a cytotoxic response (155). Interestingly they have shown that the MET positive cells are preferentially located in the vascular niches and they are resistant to radiation and highly tumorigenic (194). Altogether these results underline the importance of MET status evaluation in EGFR-directed approaches and support the necessity for synergistic therapies.

Hypoxia and cell metabolism. As we have mentioned above there is an important relationship between EGFR and tumor cell metabolism, which could be particularly relevant in the GBM niche context. A histopathological hallmark of GBM (particularly the primary ones) is the presence of large fields of necrosis, which are related to a worse outcome (195). Interestingly Steinbach and coworkers have shown that EGFR inhibitors have a protective effect against cell death induced by acute hypoxia, opposite to their pro-apoptotic effects under normoxia (196). In hypoxic conditions cells generate metabolic adaptative responses such as reduced synthesis and increased nutrient catabolism. The mTOR protein kinase, present in the EGFR downstream signaling pathway, is known to act as a nutrient and energy sensor, and the S6 ribosomal protein functions as an effector of altered translation of genes implicated in metabolic control. It has been proposed that under low oxygen conditions, EGFR inhibition reduces glucose intake, delays ATP exhaustion and maintains the integrity of the mitochondrial membrane potential, probably throw the desphosphorylation of ribosomal protein S6. The authors hypothesize that EGFR inhibition may simulate a nutrient deprivation situation, preparing cells to the low oxygen and starving conditions (196). According to these authours EGFR-TKIs could be counteractive in a highly necrotic context. However, despite the relevance of this hypothesis, a possible correlation between hypoxic and/or necrotic markers and lack of response to EGFR inhibitors is still missing.

Kinase independent functions of EGFR

Studies on EGFR have been focused mainly on the conventional signal transduction pathways. However, it has long been known that many functions of EGFR require other mechanism besides those early transient responses. In fact compelling evidence indicates that EGFR can mediate cellular processes independent of its kinase activity in several types of cancer (100). For example, the expression of a mutant EGFR receptor (D813A), with no kinase activity, is able to induce MAPK activation and DNA synthesis (197). Moreover another kinase dead EGFR mutant (K721M) can activate survival signals through the interaction with other proteins like HER2 (198;199). These kinase-independent functions of EGFR could be and additional explanation for the failure of the TKI strategies as alternatively downstream signal transducers could be regulated in a phosphorylation independent manner. Here we summarize some of the non-catalytic actions of EGFR in GBM and other cancers (Figure 3).

EGFR and glucose uptake. Weihua and coworkers have found that the receptor prevents autophagic cell death by maintaining intracellular glucose levels through interaction and stabilization of the sodium/glucose cotransporter 1 (SGLT1) (200). Interestingly EGFR-SGLT1 interaction does not respond to EGF stimulation or EGFR tyrosine kinase inhibition (201). SGLTs are capable to take up glucose into the tumor cell even against a high chemical gradient (202)

and this seems to protect the cells from apoptosis inducers. In line with these results it has been proposed that knocking down EGFR, but not inhibiting its tyrosine kinase activity, sensitizes prostate cancer cells to the apoptosis inducer adriamycin (203). Moreover siRNA mediated downregulation of EGFR also stimulated the apoptotic effect of adriamycin in liver cancer cells (204). Interestingly this apoptosis could be inhibited by increased extracellular glucose level, further supporting that intracellular glucose deficiency is a key mediator of the apoptosis sensitization induced by downregulation of EGFR (203). These same authors have demonstrated that EGFR and SGLT1 co-localized in prostate cancer tissues, and that inhibition of SGLT1 sensitized prostate cancer cells to EGFR inhibitors (gefitinib and erlotinib) (201) providing an alternative synergistic approach to cure EGFR-addicted cancers.

SGLTs are overexpressed in several tumor entities (205). Moreover, in oral squamous cell carcinoma the expression of SGLT1 has been correlated with EGFR expression (206). In these tumors an irradiation-stimulated and EGFR-mediated increase in SGLT1-generated glucose uptake, has been proposed to be required for the survival of genotoxically stressed tumor cells (207). Furthermore it has been proposed that the interaction between SGLT1 and EGFR is induced by radiation in lung cancer and that the subsequent increase in glucose uptake counteracts the ATP crisis in tumor cells due to chromatin remodeling (205). Importantly, the blockade of recovery from ATP crisis by SGLT1 inhibition may radio-sensitize tumor cells, as it has been reported in lung adenocarcinoma and head and neck squamous carcinoma cell lines (205;207). Although there are no reports of SGLT1 expression in gliomas (with or without *EGFR* amplification) this is one of the cancers with higher glucose consumption so one would expect to have an important expression of glucose transporters. In fact Flavahan and coworkers have recently shown that there is a metabolic reprogramming in GBM with more aggressive cells being able to express GLUT3, the high affinity neuronal glucose transporter allowing them to survive in nutrient restrictive environments (208). It will be interesting to test whether EGFR modulates GLUT3 stability in glioma cells. In any case it is tempting to propose a synergistic effect of an increase in glucose uptake (mediated by the stabilization of SGLT1 and/or other glucose transporters) with the PKM2-mediated glycolytic upregulation in EGFR amplified and/or mutated high-grade gliomas (72). These would reinforce EGFR addiction in those GBM and would argue for the synergistic inhibition of both, EGFR tyrosine kinase activity and receptor over-expression (or increased protein stability).

Lipid rafts activation of EGFR. TKIs targeting EGFR have also failed in breast cancer, even if the cells still depend on EGFR expression for growth. Interestingly the receptor was found to be localized to plasma membrane lipid rafts in the TKI-resistant cell lines. Lipid rafts are specialized membrane microdomains enriched in cholesterol, sphingolipids and proteins. Moreover interfering with cholesterol biosynthesis or lowering cholesterol levels synergized with gefitinib (209). These authors postulated that lipid rafts provide a platform to facilitate the interaction of EGFR, c-Src and PI3K, leading to AKT activation and pro-survival signals, independently of EGFR kinase activity (210). Furthermore in colorectal cancer there are evidences that HIF1,2 direct transcriptional activation of CAV1, an essential structural constituent of caveolae (specialized lipid raft microdomains), leads to increased dimerization and signaling of EGFR (211). However there have been no reports of lipid rafts-related activation of EGFR signaling in GBM. In fact

caveolin1 (CAV1) acts as a tumor suppressor for GBM cells. Moreover it has been proposed that caveolae-enriched cellular fractions sequesters EGFR and blocks signaling through this receptor (212). However it has been reported that EGF-induced phosphorylation of the receptor results in EGFR dissociation from caveolae whereas EGFRvIII is predominantly cytoplasmic and does not associate with CAV1 unless cells are exposed to TKIs (213). Therefore although CAV1 is overexpressed in GBM cells it seems to act as a tumor suppressor for EGFR-dependent cells (214). However it is still possible that lipid rafts (other than caveolae) activate EGFR and that cholesterol modulation of EGFR localization in the different membrane subdomains could have different survival outcomes depending on the tumor cell type or in the presence of different EGFR isoforms.

Mitochondrial apoptosis inhibition. Another kinase-independent role for EGFR in GBM survival is related to the mitochondrial control of apoptosis. Both EGFR and EGFRvIII associate with p53-upregulated modulator of apoptosis (PUMA), a pro-apoptotic member of the Bcl-2 family of proteins primarily located on the mitochondria (215). PUMA strongly induces apoptosis in colorectal cancer, malignant gliomas and in adult stem cells (216). EGFR-PUMA interaction is independent of EGF stimulation or kinase activity and induces PUMA sequestration in the cytoplasm, where it cannot initiate apoptosis. These observations are in agreement with the co-expression of PUMA with EGFR/EGFRvIII in cell lines and patient samples and with the high resistance to apoptosis inducting agents of GBMs (215).

Transcriptional activity of EGFR. Once in the nucleus EGFR can act as a modulator of transcription of several genes. In fact it has been previously described that a kinase-dead EGFR mutant can stimulate DNA synthesis in a kinase independent manner (217). Later on Lin and coworkers defined nuclear EGFR as a transcriptional co-factor that contains a transactivation domain in its C-terminus and that is able to modulate cyclin D1 gene expression (99). From then on several other transcriptional targets of EGFR have been defined, mostly implicated in cell cycle progression and the nitric oxide pathway: nitric oxide synthase (iNOS) (a protein involved in inflammation, tumor progression and metastasis) (218), B-Myb (a protein controlling proliferation) (219), cyclooxygenase-2 (COX-2) (102), aurora kinase A (a protein involved in chromosomal instability)(220), c-Myc (221), and breast cancer resistance protein (BCRP) (222). Given the fact that EGFR lacks a DNA-binding domain, mechanisms of EGFR-mediated gene regulation involve direct interaction of EGFR with STAT3 to regulate iNOS and COX2 promoters, with STAT5 for regulation of the Aurora Kinase A promoter, with E2F1 transcription factors for regulation of the B-Myb promoter, and with SRC and STAT3 to form and heteromeric complex in the nucleus that contributes to the expression of c-Myc. Constitutive presence of EGFR in the tumor nuclei may be beneficial to the tumors encountering EGFR-targeted antibodies and TKIs. In fact it has been shown that cancer cells that have acquired resistance to cetuximab (223) or gefitinib (222) expressed increased levels of nuclear EGFR. These observations provide a rationale for the combinations of inhibitors of this receptor and molecules that could block EGFR nuclear translocation like for example AKT inhibitors, as AKT-mediated EGFR phosphorylation at Ser-229 has been shown to be required for EGFR nuclear entry (222). Dasatinib, a known SRC inhibitor, has demonstrated also a synergistic effect with cetuximab by limiting EGFR translocation to the nucleus (223).

Targeting EGFR stability in GBM

If kinase-independent functions of EGFR are responsible for tumor maintenance we should look for alternative strategies that could downregulate levels of receptor, alone or in combination with TKIs. Among the different strategies that could be used to regulate EGFR protein levels the use of antisense RNAs against both wild type and mutant sequence of the receptor have been tested. This method impairs GBM cells growth *in vitro* and *in vivo* (224; 225;226), however the absence of efficient and specific siRNA delivery tools hampers the possibility of its immediate application in the clinic. Figure 4 reviews the mechanisms that control EGFR downregulation and its implication for the development of new GBM therapies. The downregulation of EGFR signaling entails a variety of cellular processes such as receptor ubiquitination, dephosphorylation, depletion of ligand access, receptor trafficking to the lysosome and its subsequent degradation (227). As we have previously mentioned CBL is the primary E3 ubiquitin ligase that is recruited to EGFR after ligand stimulation. CBL can bind directly to phospho-Y1045, or indirectly via Grb2. CBL recruits E2 enzymes to its ring-finger domain to promote EGFR ubiquitination. Ubiquitinated EGFR is recognized by the ubiquitin-binding domains of epsin I, Eps15, Eps15R and Hrs/Hgs proteins, strongly associated with chlatrin (228). Clathrin-coated vesicles containing ubiquitinated EGFR fuse with early endosomes and interact with the ESCRT (endosomal-sorting complex required for transport) which guide the ubiquitinated EGF-EGFR complex to the lysosome for degradation. Internalization seems to be a kinase-independent process, which is followed by efficient recycling to the plasma membrane (229). In fact the equilibrium between degradation and recycling determines the output of EGFR stimulation. Defective endocytic downregulation of EGFR is associated with cancer. Indeed, dominant-negative forms of *CBL* are found as oncogenes in human myeloid neoplasms (230). No such mutations have been found in GBM although the 19q13 allele containing the *CBL* sequence is frequently lost in these tumors (231). Another way to manipulate the EGFR network is to maintain the level of activity just below the threshold required for CBL recruitment and receptor degradation. This is the case for several mutant forms detected in lung cancer (232) and also for EGFRvIII (153). It is also noteworthy that EGF, but not TGFα (frequently overexpressed alongside EGFR) (233) or amphiregulin (234), triggers efficient degradation of EGFR. Interestingly co-expression of TGFα drives the tumorigenic potential of EGFR for tumor initiation (154). These results suggest that GBM cells need to block EGFR receptor degradation in order to enhance the downstream signaling and promote cell growth and proliferation. In fact this property has already been used to isolate by flow cytometry the GBM cells with higher capacity to form tumors, which are the ones that express higher levels of EGFR in the plasma membrane (225).

LRIG1. Given the relevance of EGFR signaling pathway it is not surprising that the internalization process involves a variety of positive and negative regulatory loops, which are responsible of the maintenance or termination of the intracellular signal cascades triggered by EGF-EGFR interaction and fine-tuned the cellular decisions. Among these regulatory processes, EGFR

activation is responsible for the transcription of genes like *leucine-rich repeats and immunoglobulin-like domains-1* (*LRIG1*) and *mitogen-inducible gene 6* (*MIG-6*), which code for positive inducers of receptor degradation, and *Sprouty 2* (*SPRY2)*, which code for an inhibitor of EGFR internalization (235). In fact the transmembrane glycoprotein LRIG1 has been proposed as a tumor suppressor protein due to its role in EGFR degradation. LRIG1 increases the amount of CBL recruited to the EGFR, limiting its downstream signaling (236;237) and it is also involved in EGFRvIII variant degradation in GBM cells in a CBL independent manner (238). Besides, it has been shown an increased EGFR/LRIG1 ratio in gliomas when compared with normal brain tissue, suggesting that LRIG1 downregulation is connected to the tumor progression. The overexpression of LRIG1 in glioma cell cultures leads to EGFR reduction in cell surface, independently of its activation status, and triggers cell growth inhibition and impaired invasion (mediated via MAPK and AKT signaling blockade), and enhanced apoptosis through increased caspase-8 levels release (239;240). Johansson and collaborators have recently demonstrated that soluble LIGR1 (sLIGR1) has an antitumoral effect both *in vitro* and *in vivo*, promoting cell cycle arrest by downregulating MAPK phosphorylation, with no effect on EGFR levels and activation state (240). It is thought that sLRIG1 may act through other RTKs or through an RTK independent way, which makes it a promising RTKs inhibitor with wide antitumoral activity. It has been recently published a similar effect performed by gambogic acid, which activates AMP-activated protein kinase (AMPK) with the subsequent upregulation of LRIG1, having as a result EGFR signaling inhibition, GBM cells increased apoptosis and impaired tumor growth (241).

MIG-6. MIG-6 has been identified as a molecule that is induced after EGF stimulation and enhances EGFR trafficking into late endosomes/lysosomes for its degradation (242). Upon EGFR ligand stimulation, MIG-6 is recruited to the activated receptor and suppresses its downstream signaling. During the ligand-stimulated EGFR trafficking, MIG-6 interacts with STX8, a SNARE protein required for late endosomes fusion, originating a complex that leads to EGFR lysosomal degradation (243). *Mig-6* knockout mice exhibit hyperactivation of endogenous EGFR, resulting in hyperproliferation and impaired differentiation of epidermal keratinocytes (244). High-resolution genomic profile of GBM allowed the identification of a highly recurrent (13% of tumors) focal 1p36 deletion which contains *MIG-6.* Moreover the same authors demonstrated that MIG-6 expression is down-regulated in half of the GBMs tested and there is a positive correlation between the existence of and *MIG-6* genomic alterations and the presence of EGFR amplification and/or the mutant EGFR vIII in GBM samples (242). These results support its role as a tumor suppressor in GBM, especially for EGFR-dependent tumors.

SPRY2-DYRK1A. It is well known the implication of the protein SPRY-2 in EGFR stability modulation. SPRY-2 is an inducible regulator, which is phosphorylated on a conserved tyrosine residue (Y55) after EGFR activation. This phosphotyrosine acts as a docking site for the SH2 domain of CBL, and competes with activated EGFR Y1045 phosphorylation. Hence, SPRY-2 removes CBL from activated EGFR and blocks CBL-mediated EGFR ubiquitination, endocytosis and degradation, which leads to sustained receptor signaling (245;246). Although SPRY2 is a tumor suppressor in different types of cancer, it has a tumor-promoting activity in colon cancer (247). In GBM, several members of the SPRY family are included in a transcriptome module that was associated with the EGFR amplification status in GBMs (248) suggesting that they could act

as oncogenes in at least a subset of glial tumors. In relation to this it has recently been described the role of the dual-specificity tyrosine phosphorylation-regulated kinase (DYRK1A) in EGFR stability regulation in neural progenitor and GBM cells (249;250). Although the mechanisms through which DYRK1A regulates EGFR stability are not fully characterized it has been proposed that DYRK1A may act upstream SPRY-2 modulating EGFR targeting to the lysosomes. Pozo and coworkers have indicated that DYRK1A function avoids EGFR degradation and favors recycling of the receptor to the cell surface, which results in EGFR signaling enhancement and an increase in tumor progression. Moreover the expression of *DYRK1A* correlated with that of *EGFR* in GBM suggesting that this kinase is necessary for the oncogenic action of EGFR (250). The downregulation of DYRK1A activity by siRNAs resulted in the reduction of EGFR levels in *vitro* and *in vivo*, leading to self-renewal and proliferation inhibition, increased apoptosis and delayed tumor growth (250). More interestingly pharmacological inhibition of DYRK1A kinase activity also showed a clear anti-tumor effect, indicating that it could be a good target in order to induce EGFR degradation.

All the previously exposed reinforces the notion that GBM trend to stabilize EGFR in the membrane through inhibition of receptor degradation, either by downregulating positive modulators of internalization or by overexpressing negative effectors of this process. Alterations in the expression of these modulators could explain why there is not a linear correlation between EGFR protein levels and the response to anti-EGFR therapy (10-13;168). In fact several studies suggest that EGFR activity and erlotinib sensitivity can be more accurately predicted by the ratio of *MIG-6/EGFR* in different tumors and that resistance to TKIs is associated with an increase in MIG-6 expression and therefore a decrease in EGFR activity (251;252). In fact *Mig-6* knock-out cells are unusually sensitive to gefitinib (244). Therefore measuring levels of membrane EGFR or analyzing the expression of EGFR turnover modulators might be relevant to predict the response to TKIs. On the other hand it will be interesting to test if targeting EGFR could enhance the efficacy of the current strategies focused on inhibiting EGFR activity. As a proof of principle, green tea (-)-epigallocatechin-3-gallate (EGCG), a known DYRK1A inhibitor (253) showed a potent antitumor synergistic effect with erlotinib in head and neck (254) and lung (255) cancer.

Concluding remarks

EGFR was one of the first tyrosine kinase receptors to be described and linked to tumorigenesis. In GBM, alterations in the *EGFR* gene occur in almost 50% of the cases, particularly in primary tumors. Nevertheless there is a need to revise what we know about EGFR signaling in gliomas in order to fully elucidate the mechanism for its tumorigenic action. Studies on this receptor have been focused mainly on the conventional signal transduction pathways such as MAPK and PI3K, controlling cell proliferation and survival. However accumulating data indicate that this classical view is not enough to explain the complexity of cellular functions that are being associated with activation and/or overexpression of EGFR in astrocytic cells. Compelling evidence links EGFR activity with the regulation of cellular metabolism and with the adaptative responses of GBM cells to their hypoxic microenvironment. Moreover the localization of the receptor in different subcellular compartments (mainly the nucleus and the

mitochondria) seems to be important for the control of DNA damage and apoptotic responses, crucial steps for tumor initiation and survival. Crystallographic analysis has shed some light into the nature of GBM-associated EGFR mutations, indicating the prevalence of the receptor inactive state in this tumor cells. Therefore molecules that could bind to this conformation would be preferable to treat aggressive gliomas. All these studies are fundamental to orient the therapeutic targeting of EGFR in GBMs, defining better readouts of the action of EGFR inhibitors and understanding why molecules working in other tumors fail in gliomas. While we wait for the results of clinical trials with second and third generation TKIs we need to anticipate the possible compensatory mechanisms activated by other RTKs or downstream mutations that could suggest bona fide predictive markers as well as more effective synergistic approaches. Finally we have to keep in mind that the response to EGFR activation can be independent of its kinase activity, therefore targeting receptor stability could be more effective than TKIs. Hopefully this new complex and comprehensive picture of EGFR signaling in GBM will allow us to reach satisfactory clinical results for at least a subset of patients with this terrible disease.

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Figure 1

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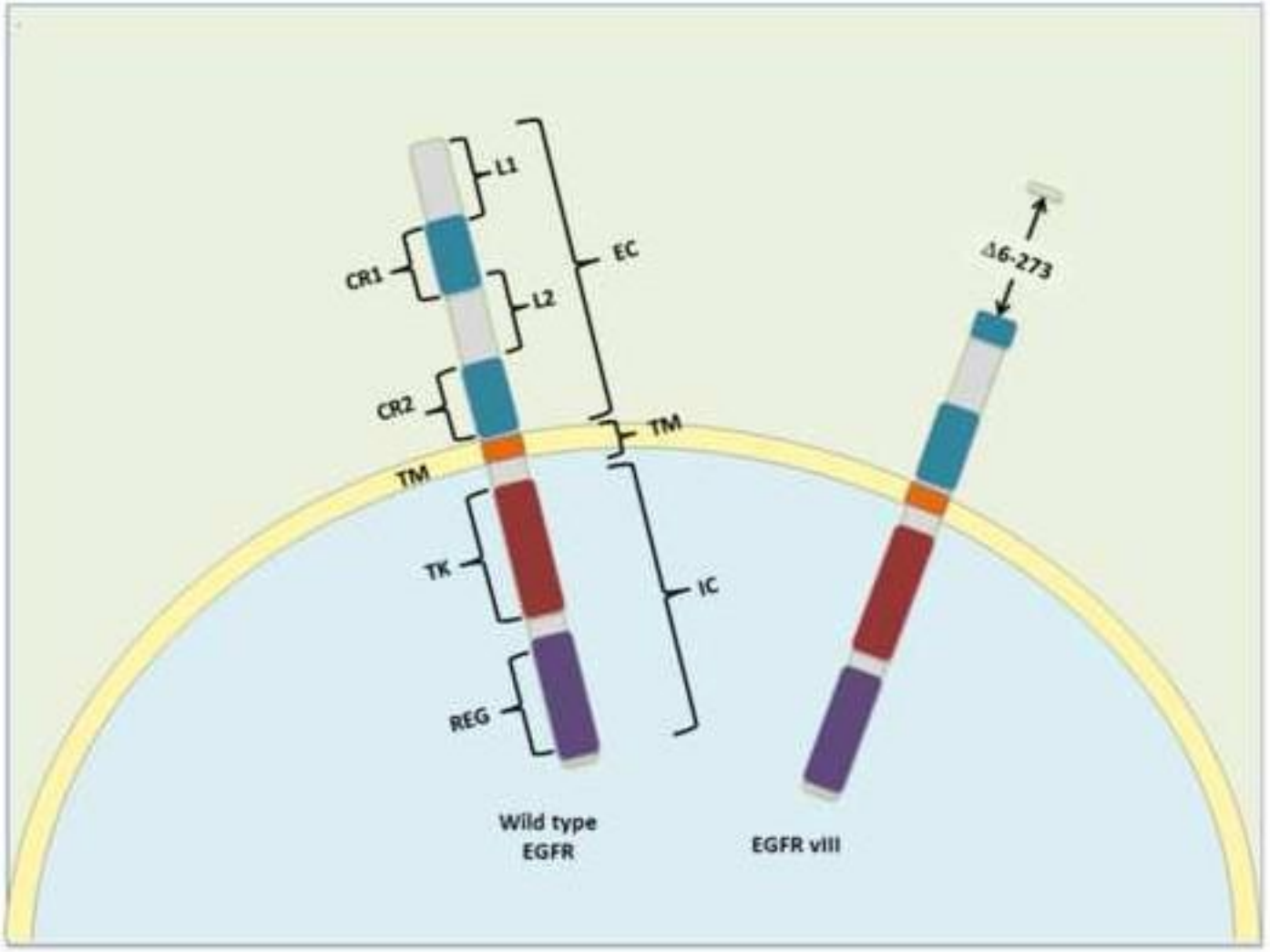

Figure 1. Structural motifs and regulatory domains of EGFR and its commonly mutated form vIII. Wild type EGFR comprises and extracellular (EC), a transmembrane (TM) and an intracellular (IC) region. The EC comprises 4 domains: L1 and L2 form the ligand-binding pocket upon folding and CR1 (cystein-rich 1) domain includes the dimerization arm. In the IC region there is a tyrosin-kinase (TK) domain and the regulatory (REG) region which includes the autophosphorylation sites and the internalization domain. In the EGFRvIII aminoacids 6 to 273 are lost and the ligand binding pocket cannot be formed.

Figure 2

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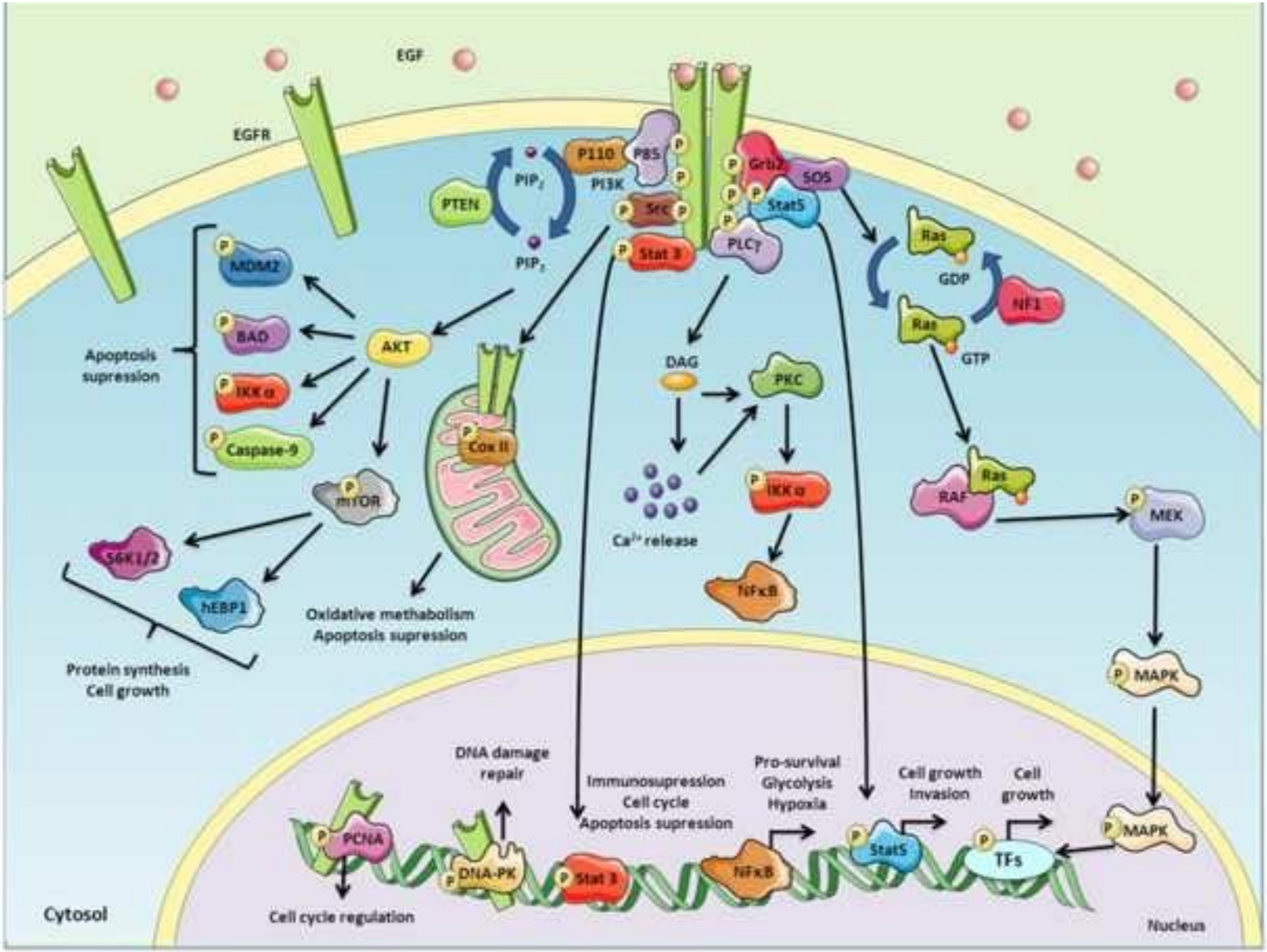

Figure 2. EGFR kinase-dependent signaling. The interaction between EGF and EGFR triggers the phosphorylation of several residues in the intracelullar domain of the receptor and the recruitment of several adapter molecules that in turn activates a variety of intracellular pathways which involves MAPK/ERK, PI3K, STAT-3, PLCγ-PKC-NFκB and Src, among other downstream signal transducers. These signaling events result in changes in protein synthesis, cell growth, apoptosis and immune suppression and cellular metabolism. EGFR can be also translocated into the nucleus, where it has an effect on cell cycle regulation and DNA damage repair.

Figure 3

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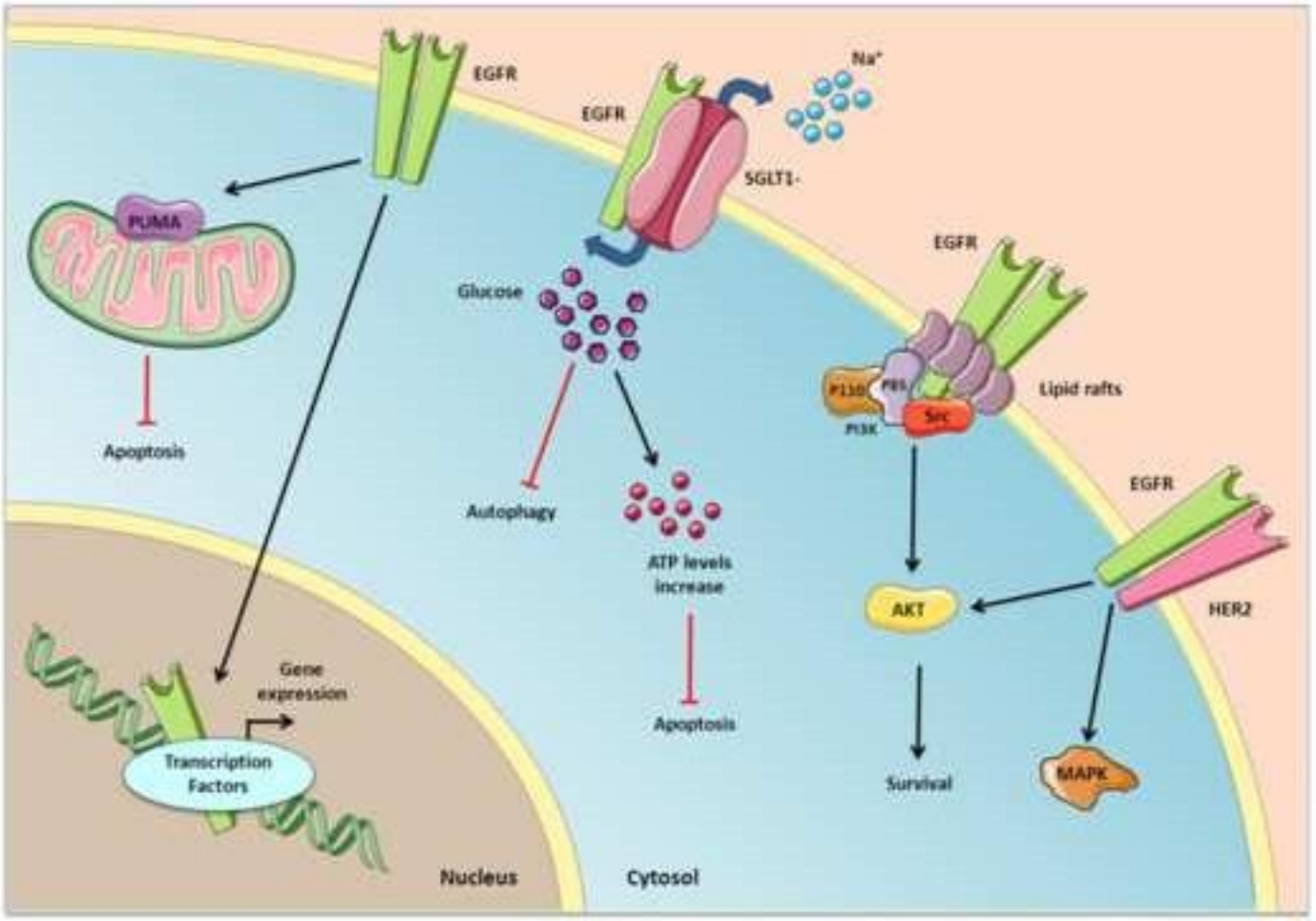

Figure 3. Kinase-independent EGFR signaling. Glucose uptake and mitochondrial-mediated apoptosis inhibition are the main processes resulting from kinase-independent EGFR signaling. EGFR can also associate with HER2 or Src and activate downstream survival signals independently of its kinase activity. Nuclear EGFR can serve as a cofactor to activate the transcription of several genes.

Figure 4

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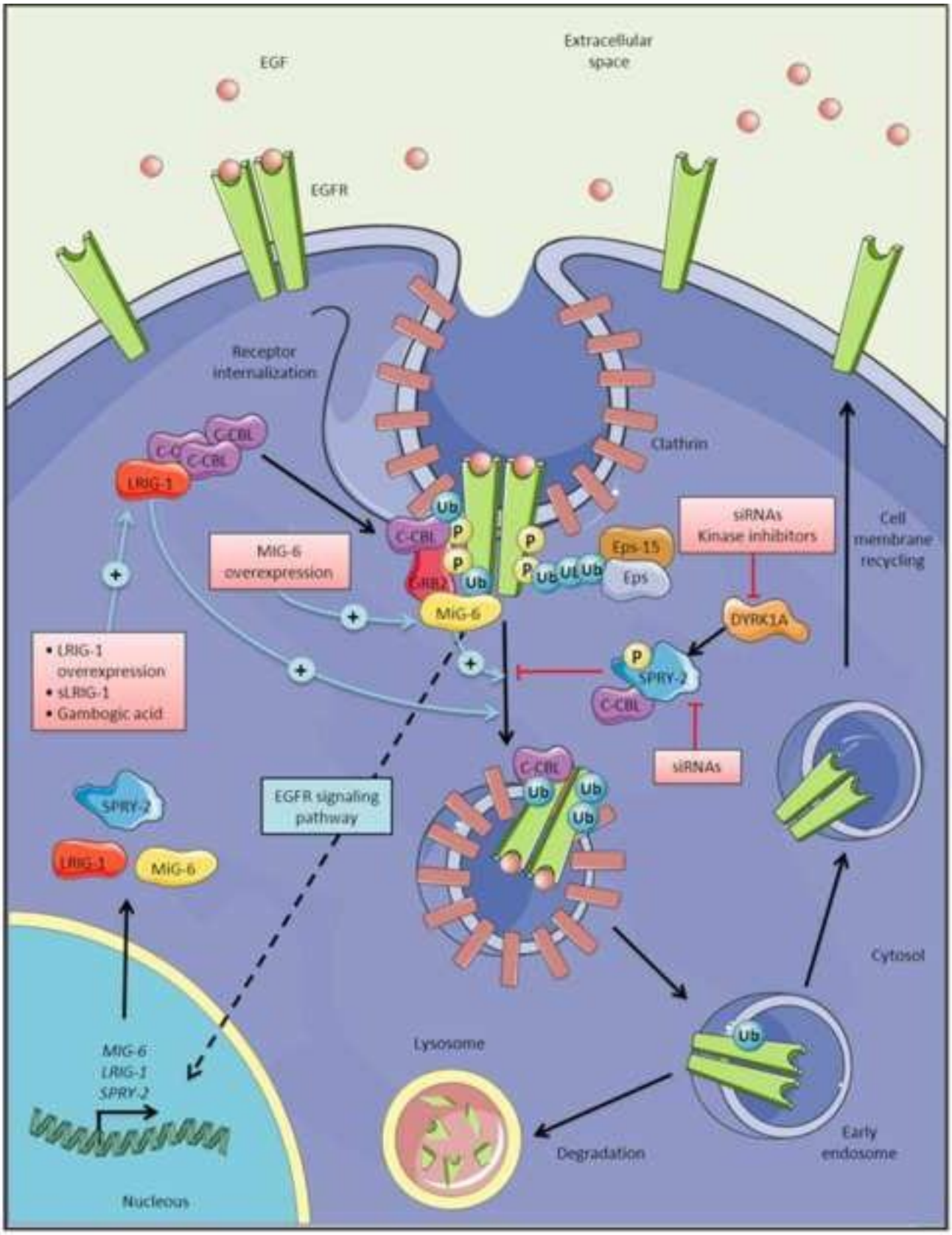

Figure 4. Regulation of EGFR turnover. Clathrin-mediated endocytosis of EGFR internalization involves a variety of proteins, which results in the degradation of the receptor in the lysosomes or its recycling to the membrane. Activation of EGFR leads to the induction of *LRIG1*, *MIG-6* and *SPRY-2* expression, all of them implicated in the regulation of EGFR turnover. Pink boxes indicate the possible strategies directed to target EGFR stability through the activation or inhibition of these proteins.