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Mechanisms of tumor resistance to EGFR-targeted therapies

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Abstract

Background—Much effort has been devoted to development of cancer therapies targeting EGFR, based on its role in regulating cell growth. Small-molecule and antibody EGFR inhibitors have clinical roles based on their efficacy in a subset of cancers, generally as components of combination therapies. Many cancers are either initially resistant to EGFR inhibitors or become resistant during treatment, limiting the efficacy of these reagents.

Objective/Methods—To review cellular resistance mechanisms to EGFR-targeted therapies.

Results/Conclusions—The best validated of these mechanisms include activation of classic ATP-binding casette (ABC) multidrug transporters; activation or mutation of EGFR; and overexpression or activation of signaling proteins operating in relation to EGFR. We discuss current efforts and potential strategies to override these sources of resistance. We describe emerging systems-biology-based concepts of alternative resistance to EGFR-targeted therapies, and discuss their implications for use of EGFR-targeted and other targeted therapies.

Keywords

ABC transporter; antibody; cetuximab; EGFR; erlotinib; network; resistance; tyrosine kinase inhibitor

1. Introduction

Therapeutic targeting of biomolecules has become a dominant theme in modern day oncology. The success achieved with imatinib [1], bevacizumab [2] and a small number of other drugs [3–5] has been of enormous clinical benefit. However, these outcomes are in stark contrast with the no less spectacular failures of many other targeted agents (e.g. gefitinib [6], farnesyl transferase inhibitors [7]). Agents targeting EGFR fall into a middle ground: some of them are extremely successful for specific types of cancer, and in a subset of patients. Better understanding of why some tumor types respond and others do not, and why some patients respond to EGFR-targeting agents and others do not, would produce vast clinical benefits. Given the particular importance of the EGFR signaling pathway in cancer, we have here

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systematically analyzed potential factors affecting tumor susceptibility or resistance to EGFR inhibitors.

The reason EGFR has been viewed a critical target involves its role as a central regulator of cell proliferation, survival and migration in normal and cancerous cells, and EGFR has long been a target of intense interest and effort for development of targeted cancer therapeutic agents. The structure of EGFR is shown in Figure 1A. EGFR is a transmembrane receptor tyrosine kinase (RTK). The extracellular, cysteine-rich domain binds activating ligands. EGFR ligands are expressed on the cell surface as tethered precursors that require proteolytic cleavage to be released to the interstitial space. A family of such proteases (sheddases, also known as a disintegrin and metalloprotease, or ADAM, proteases) release the ligands EGF, betacellulin, epiregulin, TGF-α, amphiregulin, and heparin-binding EGF-like growth factor (HB-EGF) [8]. Once released from the membrane, the 49–85-amino acid mature growth factors are able to bind the EGF receptors. In 1997, Lemmon *et al*. first suggested a model in which one EGF monomer binds to one EGFR monomer, and receptor dimerization involves subsequent obligate association of two monomeric (1:1) EGF EGFR complexes, associated with activation [9]. The intracellular domain of EGFR includes a ligand- and dimerization-activated tyrosine kinase, which upon activation autophosphorylates EGFR at residues including Y992, Y1045, Y1068, Y1148 and Y1173 [10], and also phosphorylates other binding partners, as discussed below. Cooperative physical interaction between adjacent kinase domains of adjacent erythroblastic leukemia viral (v-erb-b) oncogene homolog (ErbB) family molecules is required for EGFR activation [11,12]. The normally auto-inhibited EGFR kinase undergoes conformational changes upon allosteric contact with another EGFR kinase domain, a mechanism strikingly similar to the activation of cyclin-dependent kinases by their cognate cyclins. The increased number of EGFR molecules on the cell surface associated with overexpression in cancer is predicted to increase the rate of kinase autoactivation [12].

The intracellular domain of EGFR also encompasses a motif regulating internalization [13]. Oligomerization of the ligand-activated EGFR triggers a rapid process of receptor internalization in parallel with the autophosphorylation/activation process, which allows regulated removal of the protein from the cell surface into intracellular compartments, limiting the duration of signaling [14,15].

EGFR (also known as ErbB1) is the initially characterized member of a four-member group of proteins that also includes ErbB2/human EGFR 2 (HER2), ErbB3, and ErbB4 (Figure 1B). As part of its activation process, EGFR homodimerizes, but also in some cases heterodimerizes with ErbB2 and other family members to create an active signaling unit [16]. The four ErbB proteins have overall structural similarity, with some key differences. For example, ErbB2 is not ligand-activated, but activated based on its heterodimerization with other family members [17]; in normal cells, EGFR/ErbB2 heterodimers are more active than EGFR homodimers due to constitutive kinase activity of ErbB2, but this is regulated by the relative low expression level of ErbB2 in normal cells [18]. As another example, ErbB3 is ligand-activated, but by a different set of ligands than EGFR, and lacks an active kinase domain, so that its main transmission of signals downstream is through its heterodimeric partners [19,20].

There are three very well described and physiologically important signaling outputs of activated EGFR homo- or heterodimers:

1. EGFR activates RAS viral oncogene homolog (Ras) signaling. The adaptor protein growth factor receptor-bound protein 2 (GRB2) either directly or with assistance of another scaffold protein, v-src sarcoma viral oncogene homology 2 domain-containing protein (Shc), binds activated EGFR at auto-phosphorylated phosphotyrosines, and activates son of sebvenless (SOS), a GTP-exchange factor for Ras, initiating signaling cascades that proceed through v-raf murine sarcoma viral

oncogene homolog (Raf)/MAP kinse-ERK kinase (MEK)/extracellular signal-regulated kinase (ERK), influencing proliferation and cell cycle; v-ral simian leukemia viral oncogene homolog guanine nucleotide dissociation stimulator (RalGDS), influencing proliferation and migration; and phospho-inositol 3 kinase (PI3K), and thence to the important PI3K effector v-akt murine thymoma viral oncogene homolog (AKT), governing survival responses.

2. EGFR activates signal transducer and activator of transcription (Stat)3 and 5 leading to their phosphorylation, nuclear translocation, and transcriptional activation of genes involved in proliferative response.

3. EGFR directly binds and activates the p85 subunit of PI3K, thus providing a second stimulus to activate AKT. Although these are some of the most studied interactions, EGFR also interacts with a number of other signaling proteins; a recent peptide library screen identified multiple overlapping sites for over 40 interactions between EGFR and cytosolic proteins [10,21]. Soon after its initial discovery in 1980 [22], abnormal function of EGFR was identified as cancer-promoting, based on the discovery that the v-erbB oncogene, encoded by the erythroblastosis virus, represented a truncated form of EGFR [22–24]. The v-erbB truncation constitutively activated the tyrosine kinase activity of the protein, driving proliferation and apoptosis resistance in tumors. After three decades of study, several distinct categories of oncogenic lesions influencing endogenous EGFR activity in human tumors have been documented as clinically significant (Figure 1C). One of the best studied is activation of the EGFR signaling arising either from increased gene copy number (induced by amplification or polysomy [25,26]) or from feedback upregulation in response to cellular stresses [27–29]. Second, in some cancers, the EGFR ligands are overexpressed [30]. Third, increased activity of the EGFR signaling is mediated through activating mutations in its kinase domain, or via structural alterations of the extracellular domain such as in variant III (EGFRvIII) discussed below. These direct mechanisms of EGFR activation are observed in many different types of cancer, at significant frequencies. For example, increase in EGFR gene copy number is seen in approximately one fifth of non-small cell lung cancers (NSCLC) [31] and is found in populations partially overlapping with those possessing EGFR kinase mutations (most commonly seen in female non-smoking lung adenocarcinoma or bronchoalveolar carcinoma patients) [32]. Finally, in addition to cancers with lesions directly related to EGFR hyperactivity, EGFR and its family members are also central components of critical autocrine feedback circuits that promote growth (Figure 1C). Hence, even in cancers in which EGFR itself is not oncogenically activated, inhibition of EGFR would be predicted to have therapeutic value.

For these reasons, enormous effort has gone into the development of targeted therapeutic agents that inhibit EGFR. The most useful clinical agents fall into two classes: small-molecule tyrosine kinase inhibitors (TKIs), and EGFR-directed antibodies (Table 1). The small-molecule inhibitors of EGFR include reversible inhibitors such as erlotinib and gefitinib, and irreversible ones, such as HKI-272, EKB-569 and CI-1033. These TKIs target the intracellular kinase domain of EGFR by blocking the ATP-binding pocket thus preventing autophosphorylation on cytoplasmic tyrosines, and subsequent assembly of downstream macromolecular signaling complexes mediated through v-src sarcoma viral oncogene homology 2 domain (SH2)- or polypyrimidine tract binding protein (PTB)-domain interactions with EGFR phospho-tyrosines.

The first of the EGFR-targeting antibodies, m225, was generated in John Mendelsohn's laboratory in the early 1990's and was subsequently transformed into an acclaimed anti-cancer targeting agents, cetuximab [33]. There are presently at least five EGFR-targeting antibodies

in clinical use or under development, including cetuximab, panitumumab, matuzumab (EMD 72000), nimotuzumab (hR3), and zalutumumab [34]. Upon receptor activation, EGFR domains I and III are brought within proximity of each other, and create a binding site for the ligand molecule [35,36] (Figure 1A). Structural analyses have shown that most of the EGFR-targeting antibodies, including cetuximab, sterically hinder the interactions between ligand and binding site on EGFR [25]. Alternatively, several recently developed antibodies (e.g., 806 [37] and zalutumumab [34]) act by blocking the conformational rearrangements that are required during EGFR dimerization, while some target EGFR dimerization with partners (e.g., pertuzumab, which disrupts EGFR ErbB2 interactions [38]). Besides directly inhibiting EGFR-dependent signaling, a second action of these antibodies is to promote receptor removal from the cell surface, reducing the active pool of the protein available to signal [14]. A third, *in vivo*, activity of therapeutic antibodies is to induce antibody-dependent, cell-mediated cytotoxicity (ADCC) [39]. In this process, the Fc domain of the antibody engages the Fc receptors on macrophages and natural killer (NK) cells, driving their activation and promoting cancer cell destruction.

In rare cancers, in which there is absolute dependence of the malignant phenotype on a unique oncogenic lesion, the use of a targeted agent as a monotherapy is appropriate and yields a dramatic clinical response. This is the underlying basis for efficacy of imatinib and other drugs of its class, which block the highly potent oncogenic driver breakpoint cluster region-Abelson murine leukemia viral (v-abl) oncogene homolog (BCR-ABL), in chronic myeloid leukaemia CML [1]. For the many reasons discussed below, EGFR-targeting inhibitors are very effective as single agents only in a small subset of patients. However, because of the central role of EGFR signaling in apoptosis resistance, neo-angiogenesis, cell proliferation and damage repair, inactivation of EGFR was immediately predicted as being likely to enhance the action of cytotoxic chemotherapies, radiation or other targeted agents. EGFR inhibitors are commonly used in combination therapies with these other treatments in the clinic, and hence, in studying resistance to EGFR-targeting therapies, it is also necessary to consider interactions between EGFR and its co-administered agents.

In this review, we summarize current and emerging concepts of tumor resistance to pharmacologic signaling inhibitors, particularly bearing on resistance to EGFR. These topics include (1) pharmacokinetic resistance mechanisms, in which drugs fail to reach their targets; (2) resistance due to changes in the drug target; (3) compensatory mechanisms and lateral rescue pathways in cancer cells, that render targeting ineffective; (4) the potential for intelligent development of combinatorial strategies, that utilize understanding of biological networks to devise synergistic therapies that override resistance. Although EGFR is the main focus of this review, this discussion emphasizes commonalities that can be applied to many other pathway-targeted inhibitors.

2. Interaction of EGFR and the classic cellular machinery for drug resistance

In thinking about drug resistance in relation to inhibitors targeting EGFR, a natural tendency is to focus primarily on EGFR-centric signaling processes. However, because the primary clinical use of EGFR inhibitors is in combination with classic chemotherapeutic agents, it is important to also consider the interactions between EGFR and the ‘classic’ drug resistance machinery when evaluating mechanisms of resistance to therapies involving EGFR inhibitors.

2.1 Drug transporters

ATP Binding Cassette (ABC) transporters, contribute to resistance against chemotherapeutic agents by pumping drugs out of the cancer cell. ABC transporter group members, classified in subfamilies ABCA ABCG based on the homology in their ATP binding domains and other criteria [40], are widely expressed in tissues. ABC transporters efflux a wide range of agents, including the EGFR-targeted TKIs gefitinib, erlotinib, and lapatinib [41–43], with considerable

but incomplete overlap in drug specificity among the different ABC family members [44] (Figure 2A). These transporters limit the amount of active drug entering into some tissues, and therefore may limit *in vivo* availability of a specific signaling inhibitor.

Significantly, a growing body of experimental evidence links the activity of the EGFR signal transduction pathway to regulation of ABC transporters. A number of recent studies indicate that changes in the activity of EGFR and its effectors in cancer cells regulate the expression and activity of many transport proteins (Figure 2B). EGF-induced transient activation of EGFR transcriptionally upregulates members of the multidrug resistance protein (MRP, also known as ABCC) transporter subfamily, including MRP1 (also known as ABCC1) and MRP7 (ABCC10), in the breast adenocarcinoma MCF-7 cell line [45], compatible with the idea that active EGFR signaling may result in drug resistance [45]. Exogenous overexpression of constitutively active Ras increases expression of the important ABC transporter P-glycoprotein (P-GP, also known as multidrug resistance gene, or MDR1, and as ABCB1), and induces colchicine resistance in human and other mammalian cell lines [46,47]. Conversely, Schaich *et al*. reported an inverse correlation between activating Ras mutations and the mRNA expression of the P-GP/MDR1 transporter in acute myeloid leukemia (AML) [48]. Taken together these studies suggest a cell-type-dependent relationship between Ras and MRP1 activity.

The EGFR effector PI3K, and PI3K-activated effectors, regulate cell survival and protect against a wide range of apoptotic inducers. PI3K activation selectively upregulates transcription of MRP1 but not P-GP/MDR1/ABCB1, and selects for chemoresistant cells, in a prostatic carcinoma model [49]. A corroborating report indicates that phosphatase and tensin homolog (PTEN) phosphatase activity, which inhibits the PI3K pathway, correlates with the mRNA and protein expression levels of MRP1 and another transporter, breast cancer resistance protein (BCRP, also known as ABCG2), but does not correlate with P-GP/MDR1/ABCB1 status, in prostate cell lines [47,50]. BCRP/ABCG2 a somewhat divergent ‘half transporter’ has only one ATP binding cassette domain [51], and one transmembrane domain [52]. This is in contrast to the two ATP-binding cassette domains and two transmembrane domains contained in MDR and MRP subfamily members. Interestingly, the BCRP/ABCG2 transporter is expressed at different levels in leukemia and solid tumors samples [53] and five independent studies have correlated BCRP/ABCG2 expression to AML therapeutic response. Higher levels of BCRP/ABCG2 are found in patients that do not go into post-treatment remission, and have been linked to lower survival rates [54]. The anti-carcinogenic agent curcumin has been shown to inhibit the PI3K/Akt/NF-κB pathway, and thus downregulate the ability of P-GP/MDR1/ABCB1 to confer resistance to adriamycin [55]. Choi *et al.* suggested that this work provides direct evidence that inhibition of an EGFR effector pathway can counter the efflux capabilities of P-GP/MDR1/ABCB1, possibly by suppressing its expression [55].

EGFR signaling through Ras activates RalGDS, which subsequently triggers Ral which then recruits the Ral binding GTPase activating protein (GAP) Ral binding protein 1 (RalBP1) (also known as (Ral interacting protein, 76 kDa (RLIP76)) [56–58]. RLIP76 mediates a rather unusual connection between the Ras signaling pathway and transport activity for xenobiotics. Besides acting as a RalGAP, the ubiquitously expressed RLIP76 has the features of an unusual ABC transporter: it contains two nucleotide-binding domains, but does not contain clearly defined transmembrane domains, although it has integral membrane associations. RLIP76 confers drug resistance to anthracyclines, vinca alkaloids, and 4-hydroxy-2-nonenal (4-HNE). Hence, EGFR-initiated activation of RLIP76 can induce transport of a variety of cationic and anionic xenobiotics, including doxorubicin and vinorelbine, which have been tested successfully in combination therapy with the EGFR targeting agents lapatinib, cetuximab and gefitinib. [59–62] Separately, RalBP1 is involved in the clathrin-coated-vesicle-mediated endocytosis of important growth factor receptors, including not only EGFR, but also the insulin

and TGF-β receptors [63], connecting its action to feedback loops, as discussed in following sections.

Based on the above connections, EGFR-targeting TKIs are hence well positioned to be inhibitors of the ABC transporters. Indeed, gefitinib has been shown to function as a potent ABC inhibitor, reversing resistance to and enhancing cytotoxicity of the well known BCRP/ABCG2 and P-GP/MDR1/ABCB1 substrates, topotecan, irinotecan and mitoxantrone, in BCRP/ABCG2 or P-GP/MDR1/ABCB1 overexpressing cells [42]. As another example of its anti-pump activity, gefitinib inhibits P-GP/MDR1/ABCB1-mediated transport of its substrate calcein AM [64]. A similar range of clinically relevant anti-BCRP/ABCG2 effects was demonstrated for lapatinib, a dual inhibitor of EGFR and HER2 [43]. Furthermore, Shi *et al*. have reported that erlotinib inhibits P-GP/MDR1/ABCB1-mediated resistance to paclitaxel, colchicine and vinblastine [65]. These data all suggest that one action of EGFR pathway TKIs in the clinic involves increasing the activity of co-administered cytotoxic agents.

As noted above, EGFR-targeting TKIs are simultaneously substrates of the same transporters they inhibit [42]. For example, experiments performed in BCRP/ABCG2- or P-GP/MDR1/ABCB1-transfected cells show that both of these proteins transport erlotinib [42]. Deletions of the *P-gp/Mdr1/AbcB1* or *Bcrp/AbcG2* genes in mice result in higher levels of systemic exposure after erlotinib administration [42]. These results suggest that for therapeutic gain, erlotinib efflux could be suppressed by co-administration of P-GP/BCRP inhibitors. Such inhibitors of P-GP/MDR1/ABCB1 include verapamil, quinidine, cyclosporine A and tariquidar [66] and for BCRP/ABCG2 include tariquidar, cyclosporine A and fumitremorgin C [44]. To date, no work has addressed this possibility. Further studies are needed to better establish the role of transporters in EGFR resistance mechanisms.

As a side note, one of the most intriguing connections between specific ABC transporters and drug resistance is their recently described association with normal and cancer stem cells [67]. A so-called ‘side population’ (SP) enriched for stem cells is easily identifiable by flow cytometry in clinical samples and cell lines [67,68]. SP cells are characterized by their accelerated efflux of Hoechst 33342 and Rhodamine 123 (Rh123) fluorescent dyes. Both P-GP/MDR1/ABCB1 and BCRP/ABCG2, are highly expressed by these SP cells. One recent study has shown that BCRP/ABCG2 is directly required for the maintenance of the SP in the breast cancer cell line, MCF7 [69]. Furthermore, both BCRP/ABCG2 expression and SP presence was suppressed during TGF-β-induced epithelial mesenchymal transition. Whether signaling pathways essential for survival of majority of cancer cells are similarly active in stem cells remains be documented; however, certainly one current concept of drug resistance suggests it is dependent on the protected status of stem cells [67]. Acquired resistance to EGFR-targeted agents due to changes in cancer stem cells may be a rational avenue for future investigations, since novel stem-cell-directed agents are emerging. One of these drugs, the BCRP/ABCG2 inhibitor fumitremorgin C, selectively affects the cancer stem cell population in an NSCLC cell line [68]. Furthermore, erlotinib inhibits BCRP/ABCG2-mediated resistance to flavopyridol, SN38 and mitoxantrone, raising the possibility of a potential role of EGFR signaling in regulating BCRP/ABCG2 activities related to drug resistance in stem cells [65]. It is likely that this will be an area of intense investigation in coming years.

2.2 Survival machinery

For EGFR-targeting agents as for other targeted and non-targeted agents in cancer the execution steps of cellular apoptosis may be yet another source of cancer cell resistance. A detailed discussion of the cell death machinery in cancer therapy is beyond the scope of this review, but has recently been reviewed thoroughly by Lessene *et al*. [70]. Notably, inhibition of the EGFR pathway with erlotinib in sensitive cell lines leads to upregulation of the BH3-containing apoptosis activator proteins B-cell lymphoma protein 2 (BCL2)-interacting mediator of cell

death (BIM) and p53 upregulated modulator of apoptosis (PUMA, also known as BCL2 binding component 3 (BBC3)) [71], leading to subsequent oligomerization of BCL2-associated X protein (BAX) and BCL2-associated agonist of cell death (BAD) and formation of mitochondrial pores [72]. This pro-apoptotic effect of erlotinib, operating via the intrinsic mitochondrial pathway, can be counteracted by overexpression of BCL-2 even in the presence of effective suppression of Erk and Akt phosphorylation [71]. Gefitinib killing of NSCLC cells has also been shown to require a contribution from BIM [73]. Additional studies have tied resistance to EGFR-pathway-targeting agents such as lapatinib to increased expression of the anti-apototic myeloid cell leukemia sequence 1 (MCL-1) and reduced expression and activity of BCL2-antagonist/killer 1 (BAK) and BAX [74]. Intriguingly, one study has suggested that the intracellular domain of ErbB4 itself has properties of a Bcl-2 homology 3 (BH3)-domain protein, and communicates directly with the proteins BCL-2 and BAK to regulate apoptosis [75]. These studies clearly indicate the potential of manipulating the core cellular survival to increase potency of EGFR-targeted and other cancer therapies.

3. EGFR pathway-specific resistance mechanisms

3.1 EGFR-intrinsic factors

A major determinant of whether tumors will or will not respond to EGFR inhibitors often involves changes directly affecting the EGFR protein (Figure 1C). A minority of human cancers with hyperactive EGFR signaling are exquisitely sensitive to withdrawal of signaling inputs mediated by such specific inhibitors, because they have become pathway ‘addicted’ [76]. Certainly, the increased EGFR gene copy number observed in some tumors provides genetic evidence for the positive selection of potentially EGFR-addicted cancer clones, and in NSCLC and in colon cancer has been associated with higher clinical efficacy of EGFR antagonists, erlotinib and cetuximab [25,26,77].

Oncogene addiction, or at least increased sensitivity to EGFR-targeting TKIs, has in some cases been associated with specific mutations affecting the EGFR protein. For example, Lynch *et al* reported that somatic mutations in the kinase domain of EGFR in NSCLC impart susceptibility to the TKI gefinitib [78]. Mutations in the activation loop of the EGFR kinase (exon 21) or deletion in exon 19 constitute 40% and 50%, respectively, of all kinase domain alterations associated with sensitivity to gefitinib and erlotinib in NSCLC, and are associated with longer patient survival following treatment with specific inhibitors [32,79,80]. These mutated receptors exhibit less dependency on ligand binding for their kinase activity, and are constitutively active.

The ability of this mutant EGFR to act as an oncogene was demonstrated in a mouse model of lung cancer using ΔE746-A750 and L858R transgenic EGFR constructs, which simulate the naturally occurring lesions found in human tumors [81]. The ‘addictive’ mutations unite cancers of different histological and organ site origin and similarly confer sensitivity to relevant signaling inhibitors. For example, EGFR gene amplifications or mutations are rarely seen in the upper gastrointestinal tract malignancies however, when present, they make these cancer types similarly susceptible to the effects of the EGFR inhibitors [82–85].

Balancing the identification of mutations that sensitize cells to EGFR TKIs was the observation that tumors treated with these inhibitors eventually acquire secondary mutations within the EGFR kinase domain that induce TKI resistance. The most common resistance mutation, T790M, is associated with about 50% of cases of acquired resistance to the EGFR kinase inhibitors gefitinib and erlotinib. T790M introduces a methionine in place of a ‘gatekeeper’ threonine residue. EGFR mutants that retain sensitivity to EGFR have an approximately 10-fold greater affinity for gefitinib compared with wild type l EGFR kinase domains [86]. The acquired resistance mutation T790M only slightly (twofold) reduces the affinity of the double-

mutant receptor to gefitinib, but leads to a greater increase in competitive ATP binding and more dramatic resistance to gefitinib.

In contrast to the reversible TKIs gefitinib or erlotinib, irreversible TKIs or anti-EGFR antibodies can overcome this source of resistance, as has been shown in preclinical models [87–90]. The activating effect of kinase-mutant T790M EGFR on other ErbB-family receptors and STAT3 can be counteracted by blockade of ErbB2 with lapatinib [91], and further enhanced by the anti-EGFR antibody, cetuximab [91]. This dual EGFR inhibition strategy is now being pursued in clinical settings [92].

In addition to marked sensitivity of EGFR mutant cancers to TKIs, experience in clinical practice with gefitinib and erlotinib indicates that a significant percentage of tumors with non-mutant EGFR also respond to these compounds (about 10% of all squamous cell carcinomas of the head and neck (SCCHN) [93] and 7% of NSCLC [94]), indicating that other factors are involved. One potential contributing factor to the responsiveness of wild type cancer to EGFR inhibitors may be increased production of the EGFR ligands, which has been demonstrated in clinical samples of colorectal cancer [30] and in cell lines [95]. Increased copy number of the wild type or mutant EGFR (fluorescenece *in situ* hybridization (FISH) positivity) has been also associated with sensitivity to EGFR antagonists [31,77]. Tailoring therapy according to specific EGFR mutational status can dramatically improve the response rates in lung cancer patients treated with EGFR TKI, gefitinib. Sequist *et al*. [96] reported a 55% response rate in a small Phase II study of gefitinib in 31 patients with NSCLC selected on the premise of activating mutations in the EGFR kinase domain. The frequency of EGFR kinase mutations in large randomized studies without genotype pre-selection within the American patient population is in the vicinity of 15% (13% in the Tarceva responses in conjunction with paclitaxel and carboplatin (TRIBUTE) trial [97], 17% in the BR.21 trial [94]), overlapping with FISH positivity in ~38% of patients. The BR.21 trial showed median survival improvement of nearly 2 months (10 months versus 8.3 months) in EGFR-mutant patients treated with second line erlotinib versus placebo, respectively [94,98]. In this large study, *ad hoc* analysis showed a 27% response rate to erlotinib in patients harboring activating mutations of the EGFR versus 7% in a subgroup of patients with wild type EGFR. The efficacy of EGFR antagonists in lung cancer is further complicated by the response-favorable contribution of FISH positivity, or negative contributions of activating mutations in Kirsten rat sarcoma viral oncogene homolog (KRas) or PI3K, or loss of PTEN, to the responsiveness to EGFR antagonists. These issues are discussed in the following sections of this review.

One of the first described mutations in EGFR, producing EGFR variant III (EGFRvIII), is an in-frame deletion from exons 2 through 7 (amino acids 6–273) in the extracellular domain that results in ligand-independent constitutive activation of EGFR [99]. Although not present in normal adult tissues, this mutated form of EGFR is clonally expressed on the surface of up to 40% of glioblastomas [100], and has also been recently reported as being common in SCCHN [101]. In glioblastoma cell lines, the presence of EGFRvIII confers resistance to gefitinib that is correlating with sustained signaling activity of the EGFR and persistence of phospho-Akt [102]. In contrast to the other deletions and mutations of EGFR discussed above, the role of EGFRvIII expression in response of cancer cells to EGFR-targeting treatments is more complicated. Significantly, although tumors bearing EGFRvIII have decreased sensitivity to the reversible TKIs gefitinib and erlotinib, they are more sensitive to treatment with irreversible EGFR TKIs such as HKI-272 [103]. Likewise, cancer cells expressing EGFRvIII are less sensitive to EGFR-targeting antibodies such as cetuximab [101,104], even though the *in vitro* binding of the antibody to its site in domain III of the EGFR remains preserved [105]. One recently described monoclonal antibody, mAb-h806, is capable of neutralizing this deletion variant of EGFR, and is effective *in vitro* and *in vivo* [37]. It is plausible that mAb-h806 operates through the same mechanism as the dual ErbB inhibitors [37,106,107], by

preventing ErbB2-dependent transphosphorylation of normal or vIII EGFR. It has been suggested that structural differences associated with the vIII deletion create an open conformation of EGFR that is more available for interactions with its dimerization partners, including ErbB2. The new generation of irreversible ErbB inhibitors targeting both EGFR and ErbB2 (e.g. HKI-272 and EKB-569 [108,109]) may prevent cross-activation of the EGFR from its co-receptor, ErbB2/HER2.

3.2 Factors upstream of EGFR; ligand excess

In cancers in which EGFR is non-mutated, and maintains ligand dependence, an abnormal excess of the EGFR ligands may be a potent oncogenic driver and resistance factor for reversible EGFR-targeting TKIs. Amphiregulin overexpression contributes to the malignant phenotype of many epithelial tumors, including hepatomas [110]. mRNA analysis of a series of NSCLC tumors from patients treated with gefitinib identified increased amphiregulin expression as a resistance marker [111]. In some colorectal cancers (CRCs), excess production of the EGFR ligands epiregulin and amphiregulin correlates with longer disease control by cetuximab [30]. This may be due to a greater dependency on upstream regulation of EGFR signaling in these CRCs: in contrast to lung cancers, CRCs and gliomas do not typically harbor EGFR kinase domain mutations [112], as screening of 293 colorectal tumors revealed only one such mutation. Depletion of the EGFR receptor from the cell surface and blocking ligand binding by cetuximab [113] may be particularly effective in cancers bearing no receptor alterations.

Outcomes in ovarian cancer negatively correlate with expression of HB-EGF and a sheddase, ADAM17, which releases the ligand from its cell surface bound precursor [114]. Specific ADAM inhibitors, such as INCB3619, can be synergistic with EGFR TKIs by suppressing ligand-mediated activation of both EGFR and its co-receptor, ErbB3 [115]. Suggestively, the upregulation of upstream activators of EGFR (both sheddases and ligands) has been observed in response to cellular stresses induced by cytotoxic chemotherapies sometimes used in conjunction with EGFR inhibitors: Wang *et al*. [116] discovered HB-EGF induction and activation of NF-κB in colon and pancreatic cancer cell lines as an early response element to multiple cytotoxic chemotherapy agents. A gefitinib-resistant subclone of the highly EGFR-dependent A431 cell line was shown to upregulate IGF-R1 signaling via loss of IGF binding proteins, a mechanism that effectively increases the level of autocrine IGF [117].

Together with the studies discussed above addressing EGFR and ABC transporters, these results suggest further close connections between regulation of EGFR signaling and resistance to commonly used chemotherapies.

3.3 Lateral signaling through EGFR co-receptors

EGFR signaling activity, and susceptibility to inhibition, is governed in part by its dimerization partners. Hence, the dimerization of EGFR with ErbB2 and ErbB3 has attracted attention as a potential source for therapeutic resistance, or as a potential source of novel therapeutic targets. For example, a subset of cancers co-expressing ErbB2 and EGFR are refractory to inhibitors of a single co-receptor [118]. Lapatinib, a selective TKI designed to target both EGFR and ErbB2, has shown promise in early clinical trials in patients with EGFR/ErbB2-expressing metastatic breast cancer [119], although it is not yet clear whether the biological activities of lapatinib arise from its inhibition of both receptors, or predominantly from its activity against ErbB2 [120]. Additional agents targeting EGFR and ErbB2 are under development (e.g. HKI-272 and EKB-569 [108,109]), as are agents such as pertuzumab, that interrupt pairing between EGFR and ErbB2 [121], and pan-ErbB family inhibitors [122].

EGFR heterodimerization with ErbB3 [123], coupled with upregulation of ErbB3 [11,124] also provides cellular resistance to EGFR kinase blockade. For example, Rothenberg *et al* inactivated endogenous EGFR mRNA in the highly EGFR-dependent cell line PC9, and tested a panel of mutant EGFR constructs for ability to rescue cell survival [125]. Rescue did not require retention of autophosphorylated tyrosines on EGFR, but was strictly dependent on retention of sequences allowing heterodimerization with ErbB3. Although ErbB3 lacks a kinase domain, its activation by dimerization with EGFR promoted further activating association with the p85 of PI3K, which was sufficient to compensate for disabled EGFR-dependent signaling. ErbB3 signaling is biased towards PI3K-Akt signaling, with six discrete PI3K binding sites present on this receptor [10]; Erbb3 also signals to activate the Src protein, providing separate survival signals [126,127].

Besides dimerizing with ErbB family members, several other EGFR-related heterodimerizations are of particular note for therapeutic resistance. In cases of acquired resistance to erlotinib, dimerization between the hepatocyte growth factor (HGF) receptor c-Met [128] and ErbB3 activated survival signaling pathways involving PI3K and Src, and thus completely replaced signals from the inhibited EGFR [20]. It has become apparent that in *trans* signaling is a key feature of ErbB family. Specifically, activation of the PI3K/Akt pathway is driven predominantly through phosphorylation in *trans* of the kinase-inactive member ErbB3 [129]. This phenomenon, initially described in cell lines, was subsequently validated as occurring *in vivo* in clinical samples from lung cancer patients who acquired resistance to erlotinib over time while on therapy [130] via genomic amplification of the c-Met locus and upregulation of phospho-ErbB3. These alterations are a source of acquired resistance to EGFR antagonists although this distinction may be blurred by discoveries of a high incidence of Met amplifications (sometimes coincidental with EGFR amplifications) in the untreated NSCLC tumor tissues [128,131]. The issue may be that the tumors are heterogeneous at their origin and selective pressure of a particular drug or treatment modality enriches for a predominant phenotype/genotype [132].

Finally, interactions between EGFR and the insulin growth factor receptor (IGF-1R) can also provide resistance to TKIs targeting EGFR [133]. This interaction is specifically induced when IGF-1R-expressing cancer cells are treated with gefitinib [134]. IGF-IR activates the PI3-K/Akt pathway by signaling through insulin receptor substrate-1 (IRS-1) and activates Ras and downstream signaling proteins via direct interactions with the adaptor Shc. A negative feedback loop mechanism operating through IGF-PI3K-Akt signaling is responsible for derepression of IGF-R1 and increased Akt activity, leading to resistance to mammalian target of rapamycin (mTOR) inhibitors in clinical settings [135,136]. Cross-talk between EGFR and VEGF signaling has also been well documented *in vitro* and *in situ*, where induction of angiogenesis requires EGFR activity [137] for upregulation of both proangiogenic ligands (e.g., VEGF) and the VEGF receptors. Clinically, this relationship provides an explanation for the striking parallelism between the high degree of vascularization observed in resistant tumors and the vasculature collapse when tumors are sensitive to EGFR antagonists. Combined treatment modalities with VEGFR/EGFR dual kinase inhibitors [138] are aimed at overcoming acquired resistance to EGFR inhibitors via upregulation of alternative signaling via VEGFR1, a scenario reminiscent of that seen with Met/ErbB3 and IGF-R1.

3.4 Downstream ‘core’ EGFR effectors

Critical EGFR-dependent signaling proceeds through its proximal downstream effectors, including notably the Ras group of paralogs (K-, H-, and N-Ras). Ras protein mutations have been identified as causative lesions in more than a quarter of human cancers, with K-Ras most commonly affected [139]. At the cell membrane, Ras is activated not only by ErbB family proteins, but additional growth factor receptors including IGF-1R [133], Met [20,140], and

ErbB3 [123,124]. Ras mutations, typically affecting codons 12 and 13, result in predominantly GTP-bound, activated Ras, which constitutively activates downstream pathways of cell proliferation (e.g. RAF/MEK/ERK), motility and metastasis (e.g. RalGDS/RalA), and survival (PI3K/Akt) [141]. Because of this central signaling role, it is not surprising that K-Ras mutations have recently emerged as a major predictive marker of resistance to anti-EGFR therapies.

K-Ras mutation is associated with resistance to EGFR-targeting antibody therapy in approximately 40% of CRC [30,142,143]. Lievre *et al.* reported that among 30 participants with metastatic CRC (mCRC) treated with cetuximab, a K-Ras mutation was found in 68% of non-responding participants and none of the responding participants [144]. Analysis of a large randomized study of patients with metastatic CRC treated with panitumumab showed that those with tumors harboring K-Ras mutations had a 0% response rate and 7.4-week median time to progression (TTP) compared with a 17% response rate and 12.3-week TTP for participants with tumors without K-Ras mutations [145]. Similar results have been seen in several randomized studies to date (Table 3). In fact, the use of cetuximab in patients with K-Ras mutations may be harmful, as demonstrated by the retrospective analysis of the capecitabine, irinotecan, and oxaliplatin in advanced colorectal cancer (CAIRO)-2 study. In this analysis, the addition of cetuximab to capecitabine, oxaliplatin, and bevacizumab appeared worse than capecitabine, oxaliplatin, and bevacizumab in the K-Ras mutation group (12.5 versus 8.6 months of progression-free survival (PFS), $p = 0.043$). The trend toward worse outcome with cetuximab (against Ras mutant tumors) in combination with folinic acid, fluorouracil and oxiplatin (FOLFOX) was also seen in the smaller OPUS study, in which PFS was 5.5 months with FOLFOX/cetuximab and 8.6 months with FOLFOX alone (hazard ratio 1.83 $p = 0.02$). Thus, analysis of K-Ras mutation status appears to predict those metastatic CRC patients who will not benefit from treatment, and perhaps have a worse outcome, with the use of cetuximab or panitumumab as either single agents or in combination with chemotherapy. The K-Ras-mediated primary resistance to EGFR TKI has also been reported in lung cancer [146].

The fact that EGFR-inhibited cancers can switch to dependence on alternative receptors such as ErbB3, IGF-1R, VEGFR1, and Met (which are biased to the PI3K/AKT signaling cascade) suggests the potential importance of mutations targeting effectors in this pathway in promoting resistance to EGFR-directed therapies. The PI3K axis is often hyperactivated in tumors: PI3K gene amplification has been reported in ovarian cancer [147], while the mutational activation of the catalytic p110 subunit of PI3K have been identified in ~10–15% of all cancers [148]. Overexpression of AKT, activating mutations in the regulatory p85 and p110 subunits of PI3K, or inactivation of PTEN have been shown to confer resistance to growth factor withdrawal and promote metastatic behavior in model systems [149,150]. PTEN is itself mutationally inactivated in many tumors [151], while direct binding of Ras to the catalytic p110 subunit of PI3K is required for tumorigenesis in animal models [141]. Finally, a recent high throughput sequencing study by Wood *et al* [139] sought to define the genomic ‘mutational landscape’ of breast and colon cancers. The authors concluded that the Ras-PI3K-Akt pathway was enriched for mutations in both breast and colon cancers, while for a given type, the specific genes targeted varied between individuals.

Ongoing efforts are beginning to assess the role of these additional-pathway-directed lesions in influencing response to EGFR-targeted inhibitors. For example, the genetic deletion of PTEN in the MDA-468 breast cancer cell line was shown to be responsible for resistance to gefitinib, while reconstitution of PTEN in this cell line restored sensitivity to gefitinib and resulted in gefitinib-induced inhibition of Akt [152] (as Akt localization to the cell membrane, required for its activity, involves interaction between the Akt PH domain and the third phosphate group of phosphatidylinositol-3,4,5 trisphosphate; an interaction regulated by PTEN). For the most part, clinical studies exploring the role of these genetic changes in

resistance to EGFR-targeted therapies have not yet progressed in clinical settings to the degree that mutation of Ras has, but it is highly likely that a similar correlation with treatment resistance will be found. Other EGFR/RAS proximal proteins such as RAF and its paralog B-RAF, which is mutated in 10–15% of spontaneously arising cancers [148], or RalGDS and its effectors, should also be scrutinized as likely modulators of treatment response in clinic al settings. For example, a recent study of a panel of CRC cell lines demonstrated that cell lines that are dually PI3K catalytically active mutants or PTEN null, and Ras or BRAF catalytic mutants, are highly resistant to cetuximab, in contrast to singly mutated or non-mutated cells [153]. Extending such studies to clinical use could have rapid and powerful benefits in stratification of patients for therapy.

3.5 Alternative EGFR-centered resistance mechanisms

Although most of the functions of EGFR that have been discussed above bear on its kinase activity, EGFR also takes part in complex set of interactions in the cytosol or even in the nucleus [154,155]. Upon receptor engagement by a ligand and oligomerization, rapid changes in the cytosol ensue which involve EGFR ubitiquination and endocytosis. Within minutes of physiological ligand stimulation of EGFR in normal cells, or and in cancer cell lines susceptible to EGFR antagonists, the ligand receptor complexes are removed from cell surface [156]. Two distinct pathways regulate subsequent movement of the receptor from the endosomal compartment: clathrin-mediated trafficking leads to sustained signaling and receptor recycling, while non-clathrin-coated endosomes direct the internalized EGFR to the lysosome for degradation [156].

Hence, activation of EGFR in oncogenesis may also involve genetic or epigenetic changes affecting the rates of receptor endocytosis, lysosomal trafficking and membrane recycling [157]. Similarly, cellular changes that lead to resistance to therapies that target EGFR may influence the same processes. Such resistance mechanisms have not been investigated as intensively as the mechanisms discussed in the preceding sections. However, emerging data suggest that resistance to the anti-EGFR antibody cetuximab is linked to receptor internalization process, which is significantly impaired in resistant clones. Cetuximab-resistant SCCHN clones show abnormal EGFR persistence on plasma membranes after EGF stimulation [16]. This observation may be of more general significance, as the gefitinib-sensitive lung cancer cell line PC9 is characterized by rapid EGFR endocytosis. The endocytic trafficking is impaired in the resistant QG56 line [158]. However, contrasting data from Kwak *et al*. [159] showed increased internalization of EGFR in a treatment-resistant subclone of NCI-H1650. Together, these studies suggest that proteins that control EGFR internalization may emerge as important modulators of therapeutic efficacy, and that this mechanism requires further exploration.

4. Current targeted combination therapies and resistance

Consistent with the role of EGFR as survival factor for cancer cells, combinatorial strategies of EGFR targeting antibodies and chemotherapy or radiotherapy have been developed. In such contexts, blockade of the receptor removes the on-demand EGFR activation and prevents apoptosis-based resistance and sublethal damage repair from radiation. After the initial observation by Balaban *et al*. [160] that treatment with EGF-targeting antibodies during and after irradiation augmented cell survival, even in cells with non-mutated EGFR, the use of monoclonal EGFR antibodies to sensitize cancer cells to radiation has become one of the major breakthroughs in the treatment of SCCHN [161].

Development of combinatorial strategies (Tables 1 and 3) with EGFR-targeting antibodies such as cetuximab and chemotherapy drugs identified a number of combined approaches that are currently in broad use for the treatment of colorectal [3], head and neck [162] and lung [163]

cancers Chemotherapy agents introducing strand breaks (topoisomerase I inhibitors [164]) or DNA adducts (platinum compounds [165]) interact synergistically with cetuximab, preventing DNA repair processes regulated by EGFR [27]. Cetuximab, by blocking the ligand-to-receptor access, prevents compensatory EGFR activation and phosphorylation of STAT3 [166] in response to radiation induced damage. Work by Grandis *et al.* [167] identified STAT3 as a critical element of EGFR-induced proliferation. Strategies simultaneously targeting EGFR and STAT3 have been effective in the preclinical models of SCCHN, both increasing apoptosis and reducing B cell lymphoma like X (Bcl-xL) expression [168]. One novel pharmacological strategy that inactivates STAT3 using decoy oligonucleotides mimicking its cognate binding sequence of the DNA [169] is now entering a phase I clinical trial (ClinicalTrials.gov, NCT00696176).

It is still poorly understood why combinations of an EGFR-TKI and chemotherapy have failed to improve survival of NSCLC patients in several large randomized clinical trials, regardless of whether the drugs were given concurrently [97,170] or sequentially [171]. Enthusiasm for the adjuvant use of EGFR inhibitors has been tempered by the results of Southwest Oncology Group (SWOG) trial 0023 which treated patients with inoperable stage III NSCLC with cisplatin, etoposide, and radiation, followed by consolidation docetaxel. Patients were then randomized to maintenance gefinitib or placebo. This study unexpectedly showed a decrement in survival in the gefitinib arm of 23 months (n = 118) versus 35 months for placebo (n = 125; two-sided p = .013) [171]. At the same time, the initial report from the FLEX trial [172] (presented at the 2008 American Society of Clinical Oncology Annual Meeting, abstract #3) showed significant improvement of survival in the cetuximab plus cisplatin and vinorelbine chemotherapy arm compared with chemotherapy alone (11.3 versus 10.1 months, p = 0.0441). Preclinical models attempting to address possible antagonistic pharmacological interactions between the EGFR-TKI causing G1 arrest, and cell-cycle-active anti-microtubular agents [173] do not correlate with the obvious clinical efficacy of cetuximab dosed to attain a steady state blood level. The efficacy differences between the two classes of EGFR inhibitors may stem from the differences in modulation of inducible EGFR signaling activity via ligand upregulation (a process which is most effectively blocked with cetuximab, which competes for the ligand binding site), versus modulation of constitutive ‘addictive’ signaling of hyperactive EGFR kinase by TKIs in the minority of NSCLC patients in which both drugs are active. Alternatively, they may also reflect additional antibody-specific tumor targeting contributions, such as activation of ADCC responses. More mechanistic investigations are required.

5. Conclusion

As illustrated above and in other recent reviews [174,175], an increasingly detailed knowledge of EGFR signaling relationships has begun to allow the development of rationally designed combination therapies that have begun to improve effective use of EGFR inhibitors in clinical practice. Because of the expense and length of time required to establish new trials, a number of promising combinations predicted based on preclinical work have not yet been assessed in humans, raising the potential for significant clinical gains as these combinations move through the Phase I/Phase II process. However, incorporation of new models of cell signaling now emerging from proteomic- and systems biology-based analyses suggests that these combinations are more likely to yield incremental rather than transformative therapeutic gains. The reasons why more work is required are discussed below.

6. Expert opinion: network analysis of resistance mechanisms, to develop new combination therapies

Clearly, exploiting available information about well-defined, EGFR-associated signaling pathways provides better clinical outcomes with available EGFR-targeted inhibitors, but many

tumors remain resistant to these therapies. Suggestively, studies such as those of Kassouf et al note that in some cancers treated with gefitinib, an 'uncoupling' is observed, in which the upstream signaling proteins (e.g. EGFR) are inhibited, but downstream outputs (e.g. ERK1/2, cyclin D1) remain active because additional, undefined proteins have probably compensated for the deficiency in EGFR signals [176,177]. Efficient application of EGFR-targeting agents would clearly be enhanced if it were possible to readily identify those proteins capable of providing compensatory signaling, and blocking their activity. Recent conceptual and technical advances bearing on cellular signaling networks provide powerful new tools to achieve this goal.

6.1 Defining cell signaling networks

Since the early 1990s, new methods for detecting protein protein interactions, including the yeast and other two-hybrid systems [178], tandem affinity purification (TAP) [179,180], protein complementation [181,182], and mass spectrometry [183] have yielded complementary [184] large data sets regarding protein associations. These data have in turn been merged with large genetic datasets derived from studies of functional interactions in model organisms [185], as well as bioinformatics analyses that address issues of protein co-expression, or potential conserved interactions between protein ortholog groups from different organisms [186]. It is becoming appreciated that the classic view of linear or slightly branching signaling pathways is very far removed from the reality pertaining in cells, and Friedman and Perrimon have addressed at length the need to transition from canonical and simple pathway-centric models to instead think of signaling network models [187,188]. By some estimates, many signaling proteins interact with more than 20–30 partners, providing a dense degree of connection between 'pathways' that were predominantly thought to be independent. Using currently freely available tools for network modeling [189], it is possible to begin to think about alternative sources of resistance to EGFR-targeted therapies intrinsic in a network structure.

To introduce some key network concepts, biological networks have been constructed and topologically analyzed for a variety of species. These networks provide robust resistance to perturbations such as environmental conditions and mutations [190]. For example, a large-scale gene knockout study in yeast indicated that 50% of the total genes not only were not essential for viability, but also could be eliminated without a significant consequence for organism fitness, because other genes could functionally compensate for their loss [191]. Evolutionarily, this characteristic of functional redundancy poses a great survival advantage [192,193], as single mutations are unlikely to be lethal, but can instead provide the basis for the selection of new functions [194].

Redundancy can arise in cellular signaling networks in discrete ways [195]. Homogeneous redundancy is exemplified by the existence of numerous copies of key genes existing as backups in case one copy is disabled or lost. For example, protein paralogs may share a similar structure and at least a partially congruent function (e.g. the paralogs p130 v-crk sarcoma virus CT10 oncogene homolog-associated substrate (p130Cas) and neural precursor cell expressed, developmentally down-regulated 9 (Nedd9) both bind and activate overlapping substrates in integrin signaling networks [196]). In heterogeneous redundancy, distinctly different noninteracting proteins regulate similar functional pathways with convergent endpoints: for example, protein kinase C (PKC)-ζ provides collateral activation of the MAPK signaling pathway downstream of EGFR [197].

The concept of redundancy can be further qualified by considering network structure. Nodes and edges, representing molecules (i.e. genes, proteins, small molecules) and the interactions between them, respectively, define the constituents of biological networks. Barbabasi and Albert have examined the architectural properties of biological networks and found the degree distribution (representing the probability that a node has exactly *k* links or interactions) of nodes

in these networks is ‘scale-free’, differing from that found in random networks based on a power law distribution as compared with a Poisson distribution [198]. In a random network, the degree of each node remains close to the average degree of the network: if cells were random networks, all proteins would have approximately the same numbers of ‘partners’. However, biological networks contain many nodes with few links to other nodes and a few nodes, referred to as hubs, which are highly connected. Hubs can be broken down into two categories: date and party hubs [199]. Party hubs tend to be constitutively coexpressed with their interacting partners, while date hubs only transiently overlap in expression with partner proteins. Biologically, an example of a party hub might be a core scaffolding subunit of the ribosome [200], while a date hub might be a dynamically regulated scaffolding protein that assembles protein complexes only in specific regulated circumstances (e.g. kinase suppressor of Ras (KSR) [201]). Finally, some analyses have suggested that because of the dense connectivity of biological networks, any given protein node in the cellular network is separated by no more than about five or six edge/node ‘hops’ from any other [195].

In general, the biological networks found in normal cells do not experience systemic failure upon ‘random attack’. The odds of a spontaneous mutation hitting a hub, versus a minimally connected inessential node, are low: moreover, inactivation of an individual non-hub node will not have any general consequences for the stability of the overall network connectivity. However, if a targeted node is highly connected, not supported by a redundant paralog, and thus essential for cellular function, the result is fatal (see discussions of centrality lethality theory [202,203]. These network properties, with some further qualifications [190], have critical implications for both cancer progression and targeted cancer therapies.

6.2 Defining and targeting networks in cancer cells

Early stage cancer cells have a limited number of oncogenic lesions promoting proliferation. These lesions are likely to involve proteins within the EGFR signaling cascade, as these drive cell division and increase the pool size of tumor cells. Proteins within this cascade are highly connected [187], and at least some are likely to be hubs (although we note, at the present time, the state of biological databases does not allow for a totally reliable estimate of the number of edges for each EGFR-related node, nor whether this number is similar to or higher than those seen for other signaling systems. Although unbiased large-scale datasets are accumulating rapidly [204], much information about protein interactions is curated from the published literature [205,206], and the high degree of interest in EGFR signaling ensures some degree of sample bias). Targeted therapies (especially therapeutic monoclonal antibodies) are typically designed with specificity to only one or at most two proteins. In a cancer at a relatively early stage, a reagent targeting a hub such as EGFR or an EGFR effector, particularly supported by a cytotoxic agent, is perhaps most likely to be effective.

As tumors grow beyond an initial small size, accumulating a large population of individual cancer cells, other properties affecting network structure and robustness come into play: anti-redundancy and feedback signaling. Krakauer and Plotkin have discussed the specific selection for ‘anti-redundancy’ in larger populations (whether the populations are species, pathogens, or cells within an organism [192]). In cancers, anti-redundancy arises when checkpoints that induce apoptosis of mutationally damaged cells are removed, with the classic example being the loss of tumor suppressors such as p53 [207]. The value of such a lesion, which becomes prevalent in the population, is that it allows the rapid emergence of small clonal populations with one, two or a larger number of additional genetic changes. These multiple mutations may be slightly deleterious to the overall survival of an unselected tumor, and extremely difficult to detect within a larger tumor mass, but be highly protective of tumor growth under the selective conditions posed by targeted chemotherapy or irradiation. Certainly, many aggressive tumors are well-known to be clonally heterogenous [208,209]; in fact, even prior to treatment,

the growth of a solid tumor beyond an initial small size results in imposition of stressors such as high acidity, hypoxia and nutrient deprivation, that provide selection pressures for ability to maintain anti-apoptotic and proliferative functions in a hostile environment [210].

In this context, it is perhaps instructive that large-scale efforts to sequence 'the cancer genome' for various cancer types have identified a limited number of mutations, which target many genes already known to have pro-oncogenic function, that are found in the majority of tumors [135,136,211]. These studies also identify a much larger pool of mutations occurring rarely in tumors [139,212]. It is likely that the focus on mutations found commonly in the bulk of a tumor mass may miss the presence of multiple mutations affecting genes not clearly known to be pro-oncogenic, and existing only in a small tumor cell compartment. In this view, a p53-mutated tumor, or any tumor that is able to survive and proliferate while simultaneously manifesting aneuploidy or other signs of genomic instability would be intrinsically likely to possess a medium- to high- number of sub-populations colonizing different microenvironmental niches, analogous to the diversity of Darwins' finches exploiting different macroenvironments [213].

In addition to potential genetic modification of networks, feedback loops are epigenetic mechanisms for signaling control that vary signaling input to control output levels, based on signaling sensors that gauge system output in reference to benchmark levels. Both negative and positive feedback loops exist in biological networks. Negative-feedback loops autoregulate the system, promoting homeostasis and providing stability. In contrast, positive-feedback loops are highly sensitive and have the ability to promote an active signal independent of perturbations. However, these mechanisms that maintain normal cellular function can also introduce fragilities into the network that can be exploited during cancer. For example, in normal cells, the EGFR signaling pathway is an example of a regulated positive-feedback loop. Activated EGFR signaling through the MAPK cascade to the nucleus induces transcription of EGFR ligands, TGF-α and HB-EGF, which bind EGFR and re-initiate EGFR signaling until negative signals from other sources interrupt the loop [214] (Figure 3A). Positive feedback loops can be hijacked by tumor cells and promote uncontrolled cell growth through their self-sustaining attribute, or via loss of the negative 'stop' signals due to mutation. As another example, drug resistance to erlotinib can be thought of as a product of a negative feedback loop activating the AKT pathway, also controlled by EGFR, through IGF-IR signaling [215] (Figure 3B). Phosphorylation of IRS-1 at selective serine sites causes a decrease in IGF-IR signaling [216]. Inhibition of MAPK pathway activity via blockade of EGFR signaling decreases IRS-1 serine phosphorylation, and hence increases IGF-IR-mediated AKT signaling. This circuit is robust to both upstream EGFR inhibition and downstream (MEK) inhibition, demonstrating resilience to multiple perturbations through its ability to regulate the IGF-IR IRS-1 negative feedback loop.

If an advanced stage tumor is a product of a combination of functional redundancy of specific signaling nodes, selected anti-redundancy to identify multiple discrete clonal sub-populations, overall degree of the complexity of network structure, and auto-regulatory feedback mechanisms [190], what are the consequences for an EGFR-targeted therapy? Inhibition of EGFR, typically in combination with a cytotoxic agent, will provide feedback signals that upregulate MDRs/MRPs, limiting available drug concentrations, and will also select for any available anti-redundant (mutationally tolerant) pre-existing cell populations, to help avoid the apoptosis induced by the cytotoxic agent. The shutdown of an important hub, EGFR, will provide feedback/feedforward signals that help maintain the activation of key EGFR effectors. One elegant RNAi study has demonstrated the existence of an extensive network of previously unsuspected proteins regulating ERK signaling [187]. Following EGFR and cytotoxic treatment, these (and other) 'alternative' regulators are likely to experience epigenetic upregulation and activation, or alternatively, if the genomic instability in the tumor had

fortuitously hit one of these alternative regulators, such clones would gain a survival advantage. As discussed above, the tumor genome studies (e.g. [139]) demonstrate that multiple members of a pathway become mutationally active in tumors: the degree to this phenomenon is related to specific treatment history remains to be determined (also see discussion in [217]). Figure 4A shows interactions, found using the protein protein interaction database STRING, between proteins discussed in above sections as modulators of the cellular response to EGFR targeted inhibitors. Figure 4B is a collection of these proteins (seeds) and their 'first neighbor' interactors (proteins binding directly to members of the seed group) displayed as a graphical respresentation to highlight the dense connectivity among the seeds and their proximal networks and the complexity of the EGFR network. Potentially, the mutational or epigenetic targeting of proteins within this expanded group would also help induce recurrence of a drug-resistant tumor.

What are alternative approaches to improve resistance? In considering the EGFR network structure, it is provocative to consider the date hubs, particularly those corresponding to nodes/proteins with an expression profile demonstrating they can only be readily upregulated or expressed in cell lineages corresponding to the tumor. Among these, simultaneous blockade of two or three different hubs may so significantly limit tumor growth that it is very difficult to route signals around the blockade. Nevertheless, development of an arsenal of antibody and small molecule inhibitors to target different hubs that can be used to substitute in combination therapies as specific hub-centered blocks are circumvented would be invaluable, as would the development and facile application of robust biomarkers that report critical downstream endpoints. Although there are some provocative recent reports that the tumor stroma and other associated cells may derive from tumors, and/or contain anti-redundancy-associated mutations in proteins such as p53 [218], nevertheless the tumor-associated stroma is much less likely to be genomically unstable than the primary tumor, and hence likely to have a less flexible capacity to reprogram its network. Hence, application of therapeutic agents targeting either endothelial or stromal cells, in combination with agents targeting tumor cell signaling hubs, would be predicted to further reduce the ability of tumors to override signaling blockades.

In summary, therapeutic interventions may best be improved by thinking of the War on Cancer not as a conventional war against a limited number of well-defined foes, but as a guerrilla action against an inventive foe operating while embedded in a host population that is essentially friendly to its cause. In this view, systems biology provides the information matrix that maps out key resistance hubs, while robust networks of biomarkers provide real-time tracking of how these hubs are progressively utilized over time in response to therapeutic assaults. In this situation, development of a deep armamentarium of targeted drugs will allow a rapid response to changing conditions, and flexible use of three and four agents in combination will prevent tumors from easily evading assaults. Notably, the pre-requisites for such a scenario would be a radical change in the structure and practice of clinical trials, as well as the investment of a very large amount of capital. These are challenges for the next decade.

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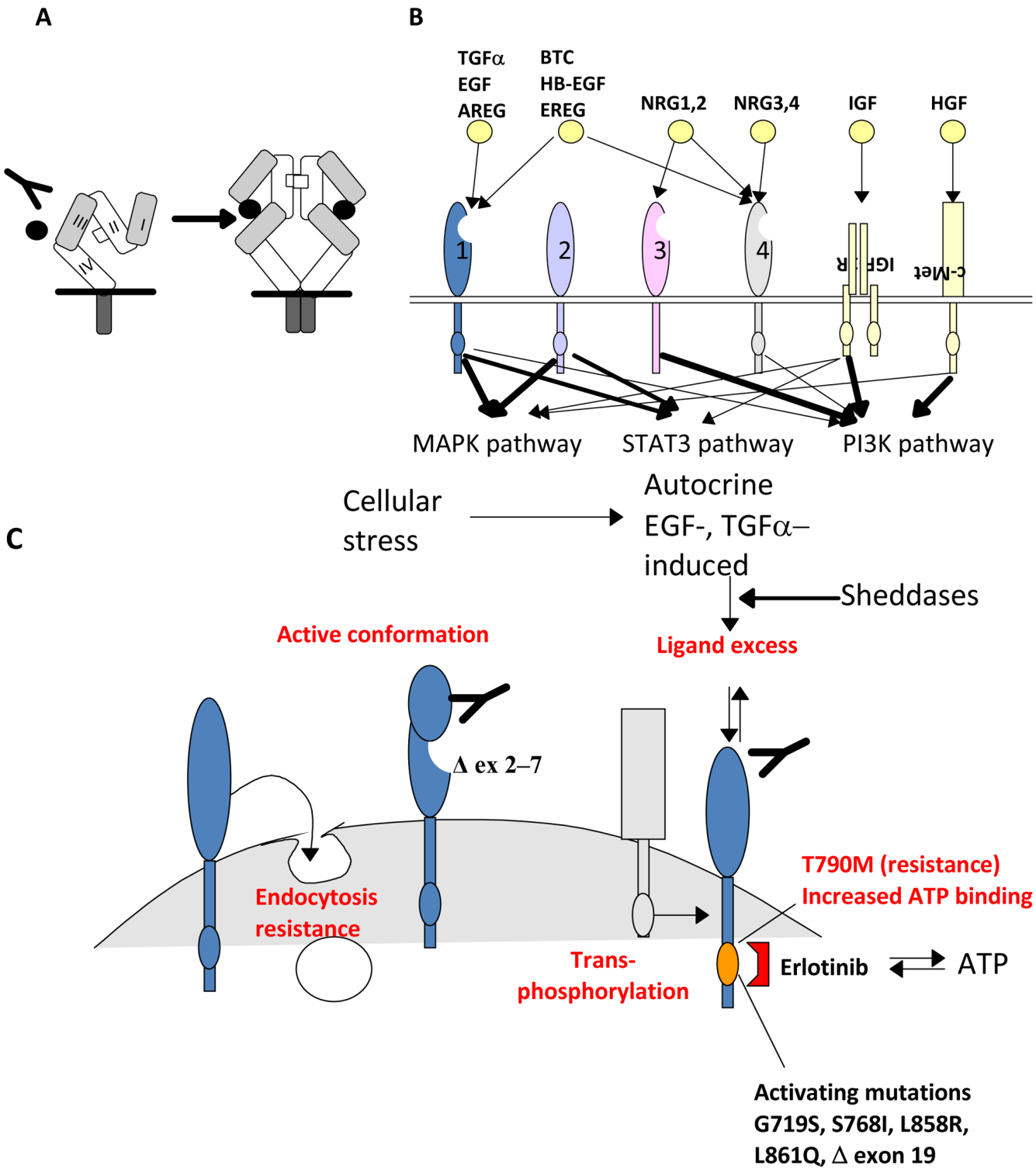

Figure 1. EGFR/(v-erb-b) oncogene homolog 1 (ErbB1) and the ErbB protein family

A, EGFR structure. Defined protein domains are numbered I-IV [113]. Binding of ligand (black circles) to domains I and III of EGFR induces unfolding from a tethered to an 'activated' conformation. The resulting exposure of dimerization domain II allows it to pair with another

EGFR molecule, or heterodimerize with another ErbB family member. Intracellular juxtaposition of the EGFR kinase domains induces trans-phosphorylation of essential tyrosine residues, activating downstream signaling. Cetuximab targets domain III, preventing ligand binding and subsequent activation steps. **B**. The ErbB receptor family, collaborating receptors, and receptor ligands [10]. EGFR homodimerizes, and heterodimerizes with all other ErbB family members [219]. All family members except ErbB3 contain a kinase domain (small ovals); ErbB3 is active in downstream signaling because of transphosphorylation. The profile of ligand binding to specific ErbB family members is indicated. AREG: amphiregulin; BTC: betacellulin; EREG: epiregulin; HB-EGF: heparin-binding EGF-like growth factor; HGF: hepatocyte growth factor; NRG: neuregulin; PI3K: phosphinositol 3 kinase; STAT :Signal transducer and activator of transcription. Signals emanating from c-Met, IGF-1R and ErbB3 modulate similar downstream outputs as ErbB family members: see text for details. **C**. Mechanisms of resistance to anti-EGFR agents. Deletion of EGFR exons 2-7 leads to constitutively active conformation of the EGFR [101,104]. EGFR kinase domain mutations, such as the common T790M mutation [86], may result in increased ATP binding and resistance to erlotinib. Receptor non-intrinsic mechanisms of resistance to anti-EGFR agents include ligand excess [115], resistance to antibody-induced endocytosis [16], or transphosphorylation [129,130].

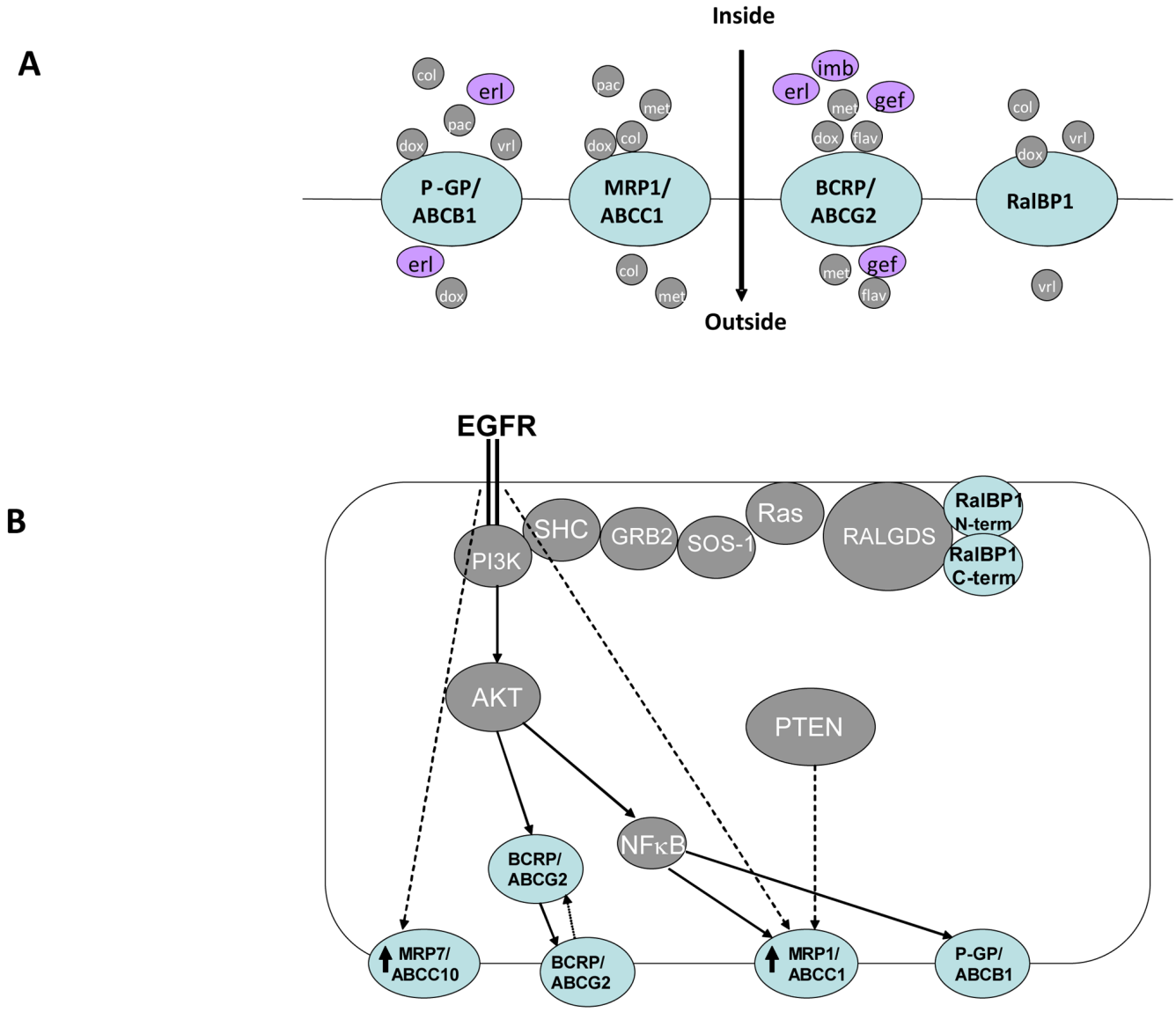

Figure 2.

A. Transported substrates of P-glycoprotein (P-GP), also known as multidrug resistance gene 1 (MDR1), and as ATP binding casette B1 (ABCB1), multidrug resistance protein 1 (MRP1)/ABCC1, breast cancer resistance protein (BCRP)/ABCG2 and Ral binding protein 1 (RalBP1)/Ral interacting protein, 76 kDa (RLIP76). ABC transporters efflux a wide range of xenobiotics from the cell. Among these, erlotinib (erl), gefitinib (gef), and imatinib (imb) target EGFR; colchicine (col), doxorubicin (dox), flavopyridol (flav), methotrexate (met), paclitaxel (pac) and vinorelbine (vrl) are cytotoxic agents commonly used in conjunction with EGFR-targeted therapies. **B. EGFR signaling mechanisms that regulate efflux pumps.** EGFR signaling pathways regulate the expression of the P-GP/MDR1, MRP1, BCRP/ABCG2 and RalBP1/RLIP76 transporters. At least three ABC transporters are regulated by EGFR via the phosphinositol 3 kinase (PI3K)–v-akt murine thymoma viral oncogene homolog (AKT) arm of the EGFR signaling pathway; phosphatase and tensin homolog (PTEN) and NF-κB contribute to this regulation. GRB2: growth factor receptor-bound protein 2; RALGDS: v-ral simian leukemia viral oncogene homolog guanine nucleotide dissociation

stimulator; Ras: RAS viral oncogene homolog; SHC: v-src sarcoma viral oncogene homology 2 domain-containing protein; SOS-1: son of sevenless homolog 1.

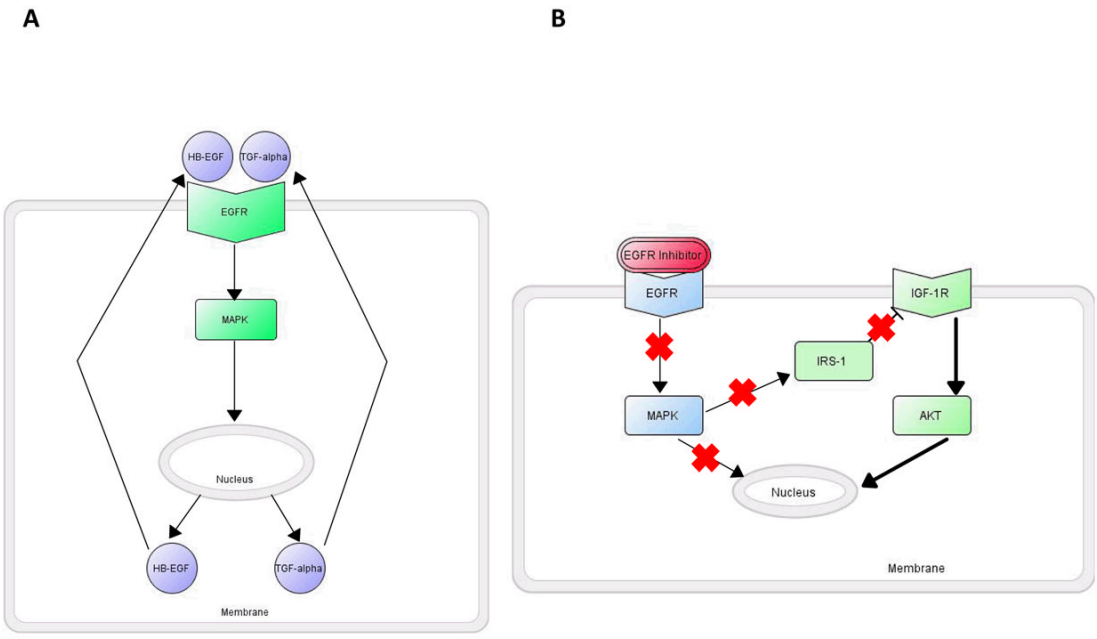

Figure 3. Feedback signaling loops modulate EGFR signaling

A. Positive feedback. In this example, EGFR-dependent activation of transcription of the genes for the EGFR ligands heparin-binding EGF-like growth factor (HB-EGF) and TGF-α (output) augments the signaling input. **B**. Negative feedback. Ligand activation of EGFR activates MAPK downstream signaling. Activated MAPK activates insulin receptor substrate 1 (IRS1), which provides negative feedback that inhibits signaling from the IGF-1 receptor. Blockade of EGFR cancels this negative feedback, and induces compensatory activation of IGF-1R. IGF-1R activates PI3K and v-akt murine thymoma viral oncogene homolog (Akt) leading to apoptosis resistance [220].

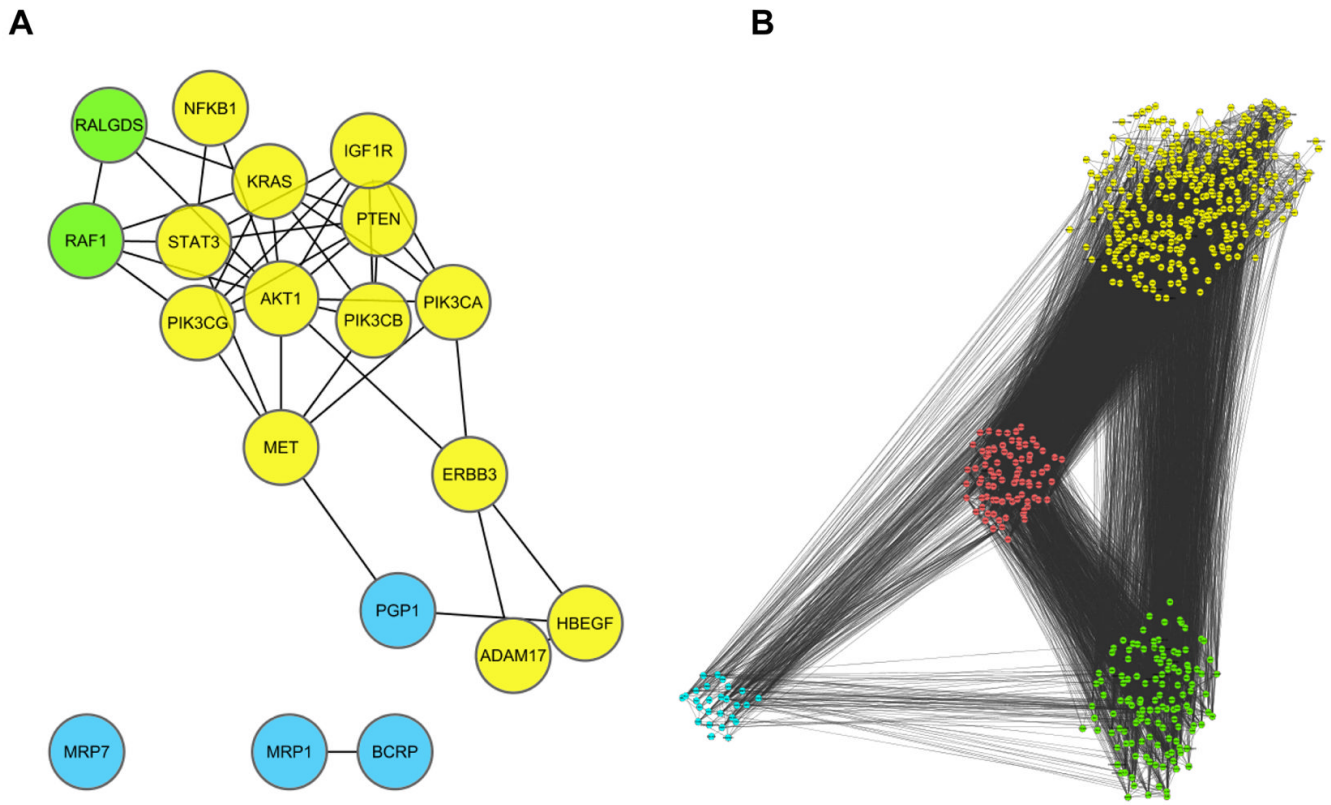

Figure 4. Networks of proteins confer resistance to EGFR-targeted inhibitors

A. Known (yellow) and potential (green) signaling proteins that regulate resistance to EGFR inhibitors. Blue, canonical transporter proteins involved in drug resistance. Analysis of these proteins in Cytoscape [221] reveals numerous direct physical interactions (lines) involving many of these proteins. ADAM: a disintegrin and metalloprotease; AKT: v-akt murine thymoma viral oncogene homolog; BCRP: breast cancer resistance protein; ERB3: v-erb-b oncogene homolog 3; HB-EGF: heparin-binding EGF-like growth factor; KRAS: Kirsten rat sarcoma viral oncogene homolog; MRP: multidrug resistance protein; P-GP: P glycoprotein; PI3K: phosphinositol 3 kinase; PTEN: phosphatase and tensin homolog; RAF: v-raf murine sarcoma viral oncogene homolog; RALGDS: v-ral simian leukemia viral oncogene homolog guanine nucleotide dissociation stimulator; STAT :Signal transducer and activator of transcription. **B**. Cytoscape was used to retrieve the set of direct binding partners for the 19 EGFR resistance modulator proteins shown in **A** (a total of 549 proteins), then map physical interactions between these direct interactors (black lines). Yellow, green, and blue represent either the initial ‘seed’ groups shown in A, or their direct interactors; proteins indicated in red interact with two or more of the ‘seeds’. The network is highly connected, involving 8537 interactions.

Table 1

Current use of anti-EGFR agents in clinical settings

Class	Synergy in clinical use
Antibodies (cetuximab panitumumab)	Cetuximab plus radiation: improved cure rate and survival in squamous cell carcinomas of the head and neck (SCCHN) [161]. Cetuximab plus platinum-based chemotherapy: improved response rate and progression free survival (PFS) in metastatic/recurrent SCCHN [162,222]; improved survival in metastatic non-small-cell lung cancer (NSCLC) [223]. Cetuximab plus irinotecan or oxaliplatin: improved PFS in patients with metastatic colorectal cancer (mCRC) [3,224,225].
Small molecule kinase inhibitors (Erlotinib Gefitinib Lapatinib)	With gemcitabine, mild survival gains (0.33 months) in metastatic pancreatic cancer. Erlotinib is approved by the FDA for treatment of pancreatic adenocarcinoma in combination with gemcitabine [226]. So far, limited benefit has been seen in combinations of tyrosine kinase inhibitors (TKIs) with radiation in early phase clinical trials marked by increased toxicity. Both gefitinib and erlotinib are antagonistic with some forms of chemotherapy in Phase III trials [97,170]. Lapatinib improves time to progression of human EGF recepter 2 (HER-2)-positive metastatic breast cancer patients [119].

Table 2

Consequences of activating Kirsten rat sarcoma viral oncogene homolog (KRas) mutations on treatment outcomes in metastatic colorectal cancer (mCRC).

Trial	Size	Treatment	PFS* (months)/Responses(%)	
			KRasWT	KRasMut
CAIRO2 [225]	755	CapOxBev	−C : 10.7 months†	−C:12.5months
		±Cetuximab(C)	+C: 10.5 months	+C:8.6months,
			p = 0.1	p = 0.044
CRYSTAL [224]	540	1st line	−C: 8.1 months/43%	−C: 8.7 months/40%
		FOLFIRI	+C:9.9 months/59%	+C:7.6 months/36%
		±Cetuximab(C)	p = 0.017/0.002	p = 0.47
OPUS.[229]	337	1st line	−C: 7.7 months/37%	−C:8.6 months/49%
		FOLFOX	+C:7.2 months/61%	+C:5.5mo/33%
		±Cetuximab(C)	p = 0.01	p = 1

*PFS (progression free survival) here defines time during which no tumor growth was detected radiographically; responses here are the sum of complete and partial responses.

†The CAIRO2 study demonstrated no difference in PFS (shown above), response (40.6% versus 43.9%, p = 0.44) or median OS (20.4 versus 20.3 months, p = 0.21) upon adding cetuximab to capecitabine, oxaliplatin and bevacizumab. In KRas mutant colon cancer patients, the detrimental effect of the two-antibody combination reached statistical significance. A similar trend was reported in the OPUS study.

Table 3

Rational synergies under exploration with EGFR inhibitors.

	Preclinical co-targets	Trials
Target	**Rationale**	**Phase**
VEGF/VEGFR	VEGF signaling confers resistance by activating PI3K-Akt [137,138]	II
ADAM17	Inhibition of sheddases prevents release of ligand [8,115]	I
ErbB3	Antibody to ErbB3 blocks survival signals to PI3K-Akt [20]	I
c-Met, IGF-1R	Antibodies and TKIs prevent transphosphorylation and PI3K-Akt signaling [20,220]	I
STAT3	Decoy oligonucleotide blocks STAT3-mediated activation of transcription, resistance to apoptosis [169]	II
mTOR	Synergistic effects in preclinical models [227], tolerated combination in mCRC	I
PI3K	Synergistic with EGFR inhibition, PI3K activation via other surface receptors (ErbB3, IGF-R1, Met) protects from apoptosis [55,228]	I
Src	Dasatinib, a multitargeted kinase inhibitor, blocks Src activity and increases efficacy of Cetuximab. Src activates signaling of ErbB family receptors [127]	

ADAM: a disintegrin and metalloprotease; Akt: v-akt murine thymoma viral oncogene homolog; ERB3: v-erb-b oncogene homolog 3; mCRC: metastatic colorectal cancer; mTOR: mammalian target of rapamycin; PI3K: phosphinositol 3 kinase; PTEN: phosphatase and tensin homolog; Src: v-src sarcoma viral oncogene homolog; STAT3 :Signal transducer and activator of transcription 3; TKI: tyrosine kinase inhibitors.