The Epidermal Growth Factor Receptor: Structure-Function

Informing the Design of Anticancer Therapeutics

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"I am both amazed and encouraged by the unpredictable scientific and clinical consequences of simply wondering what caused precocious eyelid openings in newborn mice." – Stanley Cohen[1]

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Abstract

Research on the epidermal growth factor (EGF) family and the family of receptors (EGFR) has progressed rapidly in recent times. New crystal structures of the ectodomains with different ligands, the activation of the kinase domain through oligomerisation and the use of fluorescence techniques have revealed profound conformational changes on ligand binding. The control of cell signaling from the EGFR-family is complex, with heterodimerisation, ligand affinity and signaling cross-talk influencing cellular outcomes. Analysis of tissue homeostasis indicates that the control of pro-ligand processing is likely to be as important as receptor activation events. Several members of the EGFR-family are overexpressed and/or mutated in cancer cells. The perturbation of EGFR-family signaling drives the malignant phenotype of many cancers and both inhibitors and antagonists of signaling from these receptors have already produced therapeutic benefits for patients. The design of affibodies, antibodies, small molecule inhibitors and even immunotherapeutic drugs targeting the EGFR-family has yielded promising new approaches to improving outcomes for cancer patients. In this review, we describe recent discoveries which have increased our understanding of the structure and dynamics of signaling from the EGFR-family, the roles of ligand processing and receptor cross-talk. We discuss the relevance of these studies to the development of strategies for designing more effective targeted treatments for cancer patients.

Keywords EGFR-Family; EGF-Family; Membrane Dynamics; Tyrosine kinase; Signaling; Targeting

Contents:

1. Introduction

The Epidermal Growth Factor Receptor (EGFR) is a trans-membrane protein implicated in a wide range of developmental biology processes;[2-4] and human cancers including glioblastoma, non-small cell lung cancer (NSCLC), head and neck cancer and colorectal cancer.[5-12] The EGFR family has four homologous members:[13,14] EGFR, also known as ERBB1 or HER1, HER2, also known as ERBB2, HER3 or ERBB3, and HER4 or ERBB4. Each family member has an extracellular domain (ECD) with two cysteine-rich regions, a single trans-membrane, or membrane-spanning region, a juxtamembrane cytoplasmic domain, and an intracellular kinase (or pseudokinase) domain with multiple C-terminal tyrosine residues which are phosphorylated on ligand binding and receptor activation (Figure 1).[13,15]

This article provides a framework for understanding new structural insights into the function of the EGFR and describes how new discoveries are informing efforts to improve personalised cancer treatment. We describe the structure, function and targeting of the EGFR family, starting with the extracellular components, i.e. the ectodomains (ECD), proceeding to the transmembrane and juxtamembrane components, and finishing with the intracellular domains, including the tyrosine kinase and C-terminal domains. Some therapeutic strategies, based on our knowledge of the EGFR are considered in the context of dual-target monoclonal antibodies,[16] dual-target inhibitors,[17] ligand-targeting[18] and convection enhanced delivery.[19] The use of these agents to avoid the potential development of resistance to cancer treatment is discussed. Conformational change,[20] oligomerisation,[21] and clustering[22] of EGFR are considered as mechanisms involved in ligand-stimulated

kinase activation. This update concludes with a discussion of the relevance of the models of ligand activation, EGFR signaling and recycling for the maintenance of the tumourigenic state and the potential of this information to improve outcomes for cancer patients.

2. Structural Biology of the EGFR Family and their ligands

2.1 EGFR-Family

Growth factor signaling is a critical feature of tissue homeostasis. Since the discovery of Epidermal Growth Factor (EGF),[1] the EGF:EGF-receptor (EGF:EGFR) system has been at the forefront of our knowledge on the structure and function of growth factors, cytokines and cell biology. The EGF:EGFR system is regulated at many levels, the EGFR, the release of activated ligands from their precursors, the induction and activation of the enzymes which release the EGF-like ligands, the processes and signaling modulators on the cell membrane and near the inner surface of the membrane, the phosphatases which dampen signaling and the proteins on the coordinated signaling pathways. Our understanding of each aspect of the EGF:EGFR signaling system has progressed tremendously over the last decade. Here we focus on many of the significant recent discoveries in the EGF:EGFR field.

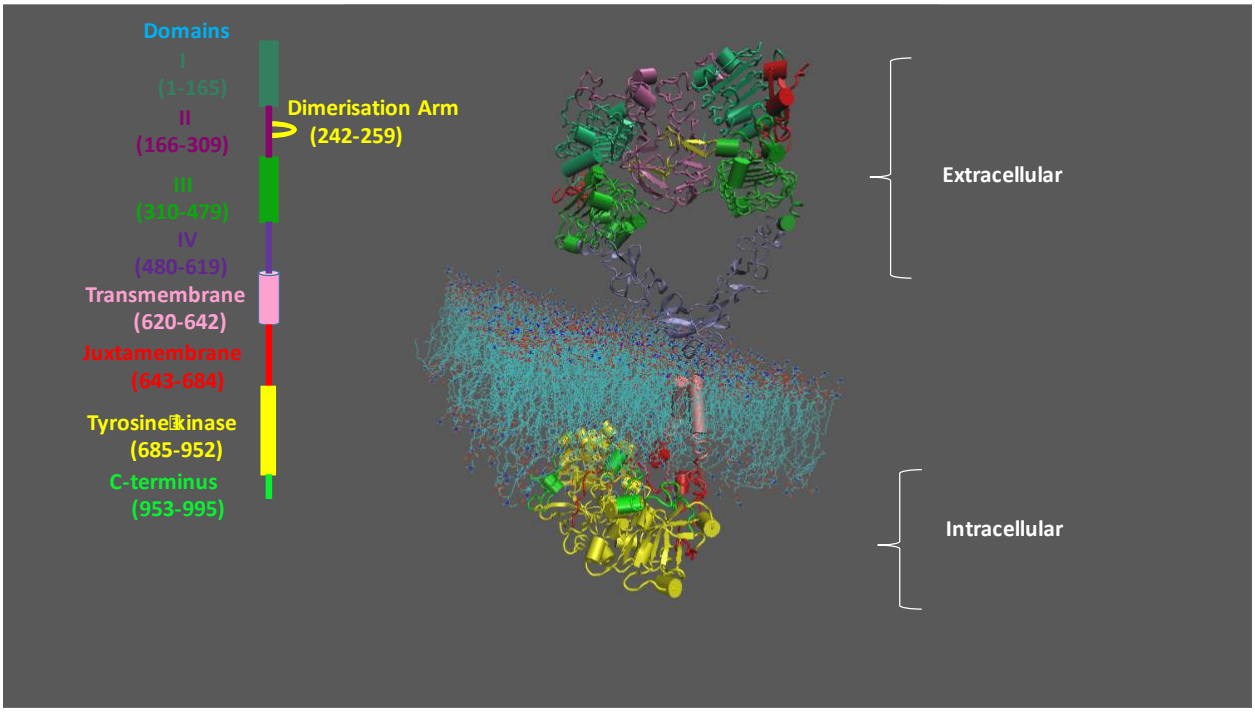


Figure 1. Schematic and three-dimensional models of the EGFR. The three regions of the EGFR including the extracellular, transmembrane, and cytoplasmic/ intracellular, domains. The extracellular component contains both ligand binding domains (I and III, or L1 and L2) is depicted in different shades of green, and cysteine rich domains (II and IV, or CR1 and CR2) in shades of purple. The lipophilic transmembrane domain, in pink, leads to the cytoplasmic region, including the juxtamembrane region, in red, and the kinase domain in yellow, and the carboxy-terminal tail in bright green. The ribbon diagram was produced from coordinates of the EGFR active dimer in Arkipov et al[23] using VMD software,[24] http://www.ks.uiuc.edu/Research/vmd/.

Activation of EGFR family members regulates many important cellular processes, such as cell division,[25] proliferation,[25] migration,[26] differentiation,[27,28] transformation,[29] and apoptosis.[15] EGF was the first EGFR ligand identified, purified and characterised.[30,31] Subsequently, 12 homologous ligands for the EGFR family including transforming growth factor α (TGF-α), amphiregulin (AREG), epiregulin (EREG),

betacellulin (BTC),heparin-binding EGF-like growth factor (HB-EGF) and the neuregulins (NRG) have been identified (see Table 1). Collectively the family of ligands bind to one or more of the four members of EGFR family.[32,33] All these ligands except the NRGs can bind the EGFR directly (Table 1).

Initial structural studies on the HER3 structure revealed a low affinity conformation, in which intramolecular interactions between extracellular domains II and IV tether each other and prevent domains I and III from coming together to form the high affinity ligand binding site.[34] This domain II-IV tether can also occur in the EGFR and again it leads to a low affinity ECD.[35] When a fragment of the EGFR containing the extracellular domains I – III (i.e. residues 1-501) is crystallised with the ligand at neutral pH a high affinity conformation (untethered) is formed.[36,37] Similar experiments at lower pH lead to crystals of the EGFR-ECD with ligand bound to the low affinity (tethered) conformation.[35] In the high-affinity conformation domains I and III of the EGFR-ECD form the ligand-binding pocket and the dimerisation loop on domain II forms homodimers with the same domain on a neighbouring receptor.[36,37] The distribution of the conformational forms of the EGFR (tethered and untethered) on the surface of living cells is yet to be described in detail.

2.2 EGF-family of ligands

A large set of EGF-like ligands control the signaling from the EGFR-family of receptors, without ligands the normal EGFR-family members do not produce a sufficient tyrosine kinase signal to activate cellular processes; without ligand stimulation any basal activity from the EGFR-family of kinases is silenced by an excess of powerful tyrosine phosphatases.[38-40] There are more than twelve active EGF-like ligands[41] (see Table1). These ligands are all small proteins capable of activating different combinations of EGFR, HER2, HER3 and/or HER4. The ligands can be considered in three classes – high affinity ligands for the EGFR

(EGF, TGFα, HB-EGF, and BTC), low affinity ligands for the EGFR (AREG, EREG, and EPGN) and the neuregulins which activate via HER3 (see Table 1).

Table 1. EGF-ligands: Structure and Function

EGF-family Ligands	RCSB PDB	UniProt	Receptor Specificity	Affinity Class	Potential Tissue /Disease Function
EGF	3NJP	P01133	EGFR	High	Wound healing
TGF-α	1MOX	P01135	EGFR	High	Renal development
AREG	2RNL	D6RFX5	EGFR	Low	Colon cancer
EREG	1K37	O14944	EGFR, ERBB4	Low	Colon cancer
HB-EGF	1XTD	Q99075	EGFR, ERBB4	High	Wound healing
BTC	1IP0	Q86UF5	EGFR, ERBB4	High	Islet cell production
EPGN	5WB8	Q6UW88	EGFR, ERBB3, ERBB4	Low	Mammary gland
NRG*	1HRE	Q02297	ERBB3, ERBB4	High	Neural cell production

*NB NRG has many isoforms

Members of the EGF-like family are made from inactive, membrane associated precursors. The active ligands are released from the precursor form and the cell membrane by the actions of metalloproteinases; all of the EGF-family members have a central β-sheet hairpin surrounded by a tightly coiled set of three disulfide bonds (Figure 2).

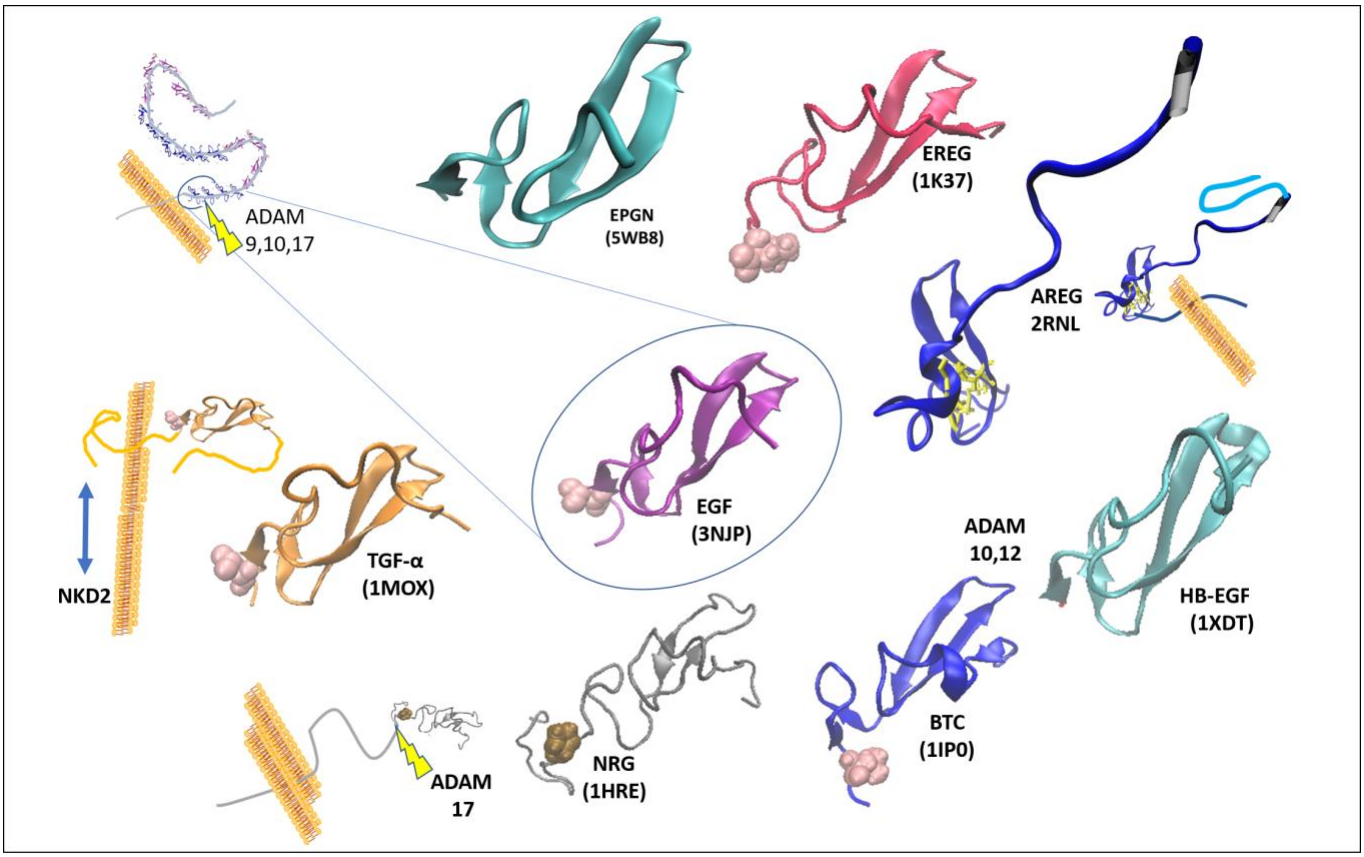


Figure 2. EGF-like ligand structures. The EGFR-family is stimulated by growth factor peptides released from larger, membrane bound pro-forms of each ligand. The ligands are not active until they are released from the pro-form by specific metalloproteases – such as members of the ADAM family. The structures are annotated with their PDB entry. The processing enzymes (ADAMS) included in the diagram are examples and do not include all of the enzymes described in the literature. The cartoon representations were generated with VMD software (http://www.ks.uiuc.edu/Research/vmd/).[24]

The EGF structural cassette is a repeated motif in many proteins including the ligand precursors, the receptors and other membrane associated signaling molecules such as the ligands and receptors of the Delta:Notch system.[42] EGF is a powerful activator of reactive oxygen species (ROS) via the activation of dual oxidases.[43] The processing of the pro-forms of the ligands requires synthesis, transport to the

appropriate location on the membrane and eventually cleavage to the activated form. It is now known that the intracellular sequences of the pro-ligands control their trafficking and localisation to the cell surface[44]. The release of the free ligand can activate receptors in a paracrine or even autocrine manner, but in some situations the ligands (e.g. AREG) are released as part of powerful exosome signaling units.[45,46] Not only do these exosome units activate receptors such as EGFR, the exosomes can target mRNA and microRNAs to change the metabolism of the target cells. Ligand such as EREG and AREG are often overexpressed in colorectal cancers (CRC). There is circumstantial evidence to suggest the excess of these ligands is likely to drive these cancers, in that the CRC patients with high levels of AREG and EREG receive more benefit from treatment with anti-EGFR antibodies than patients with low levels of the ligands.[47,48] It is still a challenge to understand how the exosome associated pro-ligands are processed before activating the receptors.

The different EGF-family members appear to have distinct roles in different tissues. Ligands such as betacellulin (BTC) can bind to and activate both EGFR and HER4, in bone over-expression of BTC can lead to an increase in bone mass.[49] In the kidney BTC is capable of activating HER4 which alters the development of the urethra.[50] BTC stimulation also leads to cross-activation of the interferon system[51] which is likely to increase the resistance of proliferating tissues to viral infections.

The third class of EGF-family ligands is the neuregulins (NRGs) which control the production and differentiation of neural cells.[52,53] The NRGs have many different isoforms, each of the isoforms can have tissue specific functions. In part the differences in function are related to the location of the specific NRG and/or different processing systems for these ligand in different cells. Some of the single membrane pass NRGs such as NRG1 and NRG2 gather on the cell surface until they are cleaved by receptor dependent activation of the cleavage metalloproteinase. Other NRGs accumulate on axons

where they are released by other proteases (e.g. BACE-1) and stimulate neighbouring neurons via HER4[54].

The activation of the ligand pro-forms can be regulated by signaling cross-talk between other ligand stimulated receptors and even the loss of homotypic cell-cell adhesion interactions. Although much of this review focuses on the structure and function of the EGFR-family, regulation of ligand activation and function[55] can have important ramifications for tissue homeostasis[56,57] and several diseases such as cancer.[58]

2.3 EGF-family Ligand activation, Tissue Homeostasis and Disease

Almost as soon as Stanley Cohen had identified the cell surface receptor for EGF[59] , he also recognized its involvement in oncogenesis.[60] The transformation of fibroblasts with the Kirsten murine sarcoma virus, i.e. by the Kras oncogene, leads to the activation and down regulation of the EGFR[60] by the induction of and activation by autocrine TGF-α.[61,62] EGF-family expression and consequential activation of EGFR-family members is a feature of tissue homeostasis[63,64], cardiac disease[65] and oncogenic transformation.[66] Although TGF-α was the first autocrine ligand of the EGFR to be described,[61,62] Axel Ullrich and his colleagues showed that particular tumours are driven by the release of HB-EGF from pro-HB-EGF:[67] the EGFR was driven by transactivation via a G-protein-coupled receptor which induced the cleavage of proHB-EGF by a metalloproteinase.

The roles of the different EGF-family members in normal tissues and cancer have been summarised in several excellent reviews[41,68,69] and recent reports.[70] There are many steps in the regulation of the expression, activation and signaling of pro-EGF-family family members[44,71]; not only do the free ligands

act as exogenous EGFR-family activators, the ligands also participate in exosomal signalling,[45] protein sorting to different cell membrane locations[44] and cross-talk with other receptor signaling systems.[72-75] Which ligands are active, the density of EGFR family members and the cell type, all dictate how long the ligand-receptor complex is activated, the endosomal organelle routing of the receptor and the proximity of the receptor to downstream effectors.[76] Wound healing leads to increased pro-EGFR-ligand expression and activation via the rhomboid[77] or ADAM proteases.[3,78-81] Interestingly, inhibitors which bind to the EGF-family ligands such as HB-EGF to prevent autocrine signaling[67] and inhibitors of the ADAM proteases[79,82,83] have potential as anti-cancer agents.

3. New approaches to ectodomain targeting – disrupting oligomerisation

Dimer or higher-level oligomer formation is important for EGFR activation.[84] The conditions for dimerisation of the EGFR have been studied in detail,[84] and it has been understood for some time that point mutations to either Y246 or Y251, two conserved tyrosines on the oligomerisation arm in domain II of the ECD, both abolish dimerisation and interfere with receptor activation.[84-86] One strategy to inhibit EGFR signaling is to target the dimerisation arm in the β-hairpin loop of Domain II of the ECD. The dimerisation arm can be targeted through a combination of monoclonal antibodies (mAbs)[87] and small molecules.[88] Traditionally mAbs have been raised against ECD targets[89] while small molecule inhibitors have been used to target the cytosolic tyrosine kinase enzyme.[90] However, computer-aided drug design is now being used to find new small molecules to target specific regions of the EGFR extracellular domain, particularly regions in domain II.[91] The goal is to disrupt the hydrophobic- and H-bond forming amino acid (aa) residues involved in dimerisation. Modelling and selection of the best hits has led to four promising new EGFR signaling antagonists DPT1-DPT4.[91] These molecules show favourable docking characteristics: all four antagonists associate with Y246, while three of the antagonists also interact with

Y251.[91] All four small molecule inhibitors depend on different functional groups to form hydrogen bonds with the aromatic ring of the relevant tyrosine residue(s), but all are predicted to have anti-oligomerisation action by preventing the participation of the β-hairpin loop of Domain II in binding to its oligomerisation partner.[91]

The mAb cetuximab binds to and blocks the ligand binding site on domain III of the EGFR-ECD.[89] Other structure-driven drug screening studies with the EGFR-ECD have identified two compounds that inhibit EGFR kinase activation, by disrupting the Y246/Y251 interface.[85] While still small molecules, these compounds are somewhat larger than DPT1-DPT4,[85,91] and while their specific binding characteristics have not been reported, it has been demonstrated that these compounds inhibit luciferase fragment complementation and EGF-induced cross linking.[85]

Another antagonist strategy has involved the development of new compounds and peptide sequences which mimic the EGFR dimerisation arm, thus blocking dimerisation, and thereby suppressing ligand-stimulated EGFR kinase activation.[92] These investigators used a cross-linked peptide sequence from the dimerisation arm of EGFR.[92] Treatment of live MDA-MB-231 cells with the cross-linked candidate peptides inhibited ligand stimulated EGFR kinase activation and consequentially EGFR autophosphorylation and downstream signaling.[92]

Using computational models of potential EGFR tetramers has resulted in the identification of molecules which bind to the EGFR and decrease internalisation. As Grb2 binding to the EGFR decreases, ligand-induced EGFR intracellular signaling also decreases.[93] Fourteen hit compounds were identified from the computer screen as potential antagonists of EGFR activation; these compounds were synthesised and tested on SCC61 cells, derived from a head and neck squamous cell carcinoma (SCC).[93] SCC61 respond to

EGF; however, none of these molecules decreased EGFR phosphorylation, nor increased cell death.[93] Perhaps the specific model of the head-to-head EGFR tetramer, to which these compounds were designed, is not the preferred oligomeric EGFR signaling assembly.

The development of targeted therapies for HER2, estrogen and progesterone receptors has improved outcomes for many breast cancer patients.[94-97] However, for triple negative breast cancer (TNBC) patients,[98] with an initially more aggressive clinical course,[98] there are no targeted therapies available at present.[99] A subgroup of TNBC patients definitely overexpress the EGFR,[100] and combinations of non-competitive EGFR-targeting therapeutics (e.g. mAb 111/cetuximab and mAb 111/panitumumab) show promising inhibition of TNBC proliferation both *in vitro*[99] and in mouse models of breast cancer.[99] All three of these antibodies displace EGF from the receptor,[101-103] and while cetuximab and panitumumab share antigenic epitopes and are unlikely to synergise, mAb 111 has a distinct EGFR epitope and shows good synergies with cetuximab and panitumumab.[99] Exposure of cells to combinations of mAbs down-regulates the EGFR profoundly: the downregulation is correlated with the occupancy of non-overlapping epitopes.[99] Cetuximab, a chimeric anti-EGFR antibody, derived from C225,[104] was the first therapeutic anti-EGFR mAb to be used in clinical trials: patients with either head and neck cancer or NSCLC were treated with the antibody alone.[105] Cetuximab binds to the human EGFR with similar affinity as the ligands, e.g. EGF or TGF-α, and therefore antagonises ligand binding.[101] The EGFR epitope for cetuximab binding has been localised to domain III of the ECD by crystallography: specifically the antibody/human EGFR contact area contains residues Q384, Q408, H409, K443, K465, I467 and N473.[89,102] Panitumumab, the first fully human anti-EGFR mAb,[106] has a similar but not identical EGFR epitope in domain III.[102] Panitumumab localises to residues W386, E388, R390, and T391.[103] In contrast mAb111 binds to R353,

R366 and H359 of domain III,[102] and this highlights the importance of targeting different epitopes to achieve therapeutic synergies.

The drug-conjugated antibody ABT-414 is another therapeutic approach which targets the EGFR with an acceptable safety profile and has had some success in glioblastoma patients. ABT-414 is a "tumour specific" anti-EGFR-antibody-drug conjugate.[107,108] It consists of a humanised recombinant immunoglobulin G(IgG)1κ antibody (ABT-806) linked to a noncleavable, maleimidocaproyl linker and a microtubule cytotoxin, monomethyl auristatin F (MMAF).[108] ABT-806 binds to a cryptic epitope on the ECD-EGFR only accessible in the vIII mutation (in which residues 6-273 are deleted and replaced by a single glycine residue),[109] or when the EGFR is amplified or overexpressed.[110] This unusual specificity means there is minimal binding of ABT-806 to normal tissue (e.g. liver), and subsequently there is less toxicity associated with ABT-414 EGFR-targeted therapy.[110,111] While ABT-414 shows frequent yet reversible ocular toxicities, the response of amplified, recurrent glioblastoma to this agent is encouraging in early studies, and a randomised trial in recurrent glioblastoma is underway.[112]

Over-expression of EGFR family members offers an opportunity to target these cancers selectively. Immunotherapy is becoming an effective option for some cancer patients and several reports have targeted over-expressed EGFR or HER2 to kill human tumour cells in mouse models. By creating a fusion protein between the double stranded binding domain from human RNA activated protein kinase (hPKR) with EGF, it has been possible to deliver polyinosinic-polycytidylic acid (poly-IC) selectively to tumour cells (MDA-MB-468) which over-express the EGFR.[113] The poly-IC was internalised by the tumour cells which died by apoptosis and secreted interferon-γ. In similar experiments targeting HER2 [113] a polyethylenimine was linked to and anti-HER2 affibody via polyethylene glycol. After forming a complex with poly-IC this reagent was able to bind to cell surface HER2 and deliver poly-IC to tumour cells over-

expressing HER2. The poly-IC not only killed the tumour cells by inducing apoptosis directly, but also induced cytokine expression, thus stimulating by-stander killing effects. As for the EGFR targeted poly-IC, this HER2 targeting system also induced an anti-tumour immune response and inhibited the growth of these tumours in mice.

4. Targeting the Transmembrane and Juxtamembrane Domains of EGFR

The 3D-structure of the EGFR transmembrane domain (TMD) was determined by nuclear magnetic resonance (NMR) spectroscopy.[114] The TMD comprises an initial N-terminal 3-10 turn (residues 644-647)[115] followed by an α-helical region (residues 648-669).[115] Mineev and colleagues[116] studied the conformation of a fragment of the EGFR comprising the juxtamembrane domains and the transmembrane domains (i.e. residues 642-690). On either side of the membrane, the juxtamembrane domains (JMDs) at the N-terminal (aas 634-642) and C-terminal (aas 673-677) of the fragment remained flexible.[116] These regions are also accessible to water.[115]

New insights into the transduction of signals involving receptors with transmembrane domains have been elicited by studying the self-association of EGFR transmembrane domain fragments in micelles.[115] In order to study the structures of the TMD and JMDs, efficient systems for production are necessary, and this has been achieved in a cell-free system for both HER2 and EGFR.[115] This production system uses the protein expression machinery from *E.coli* strains XL-1 Blue and BL21(DE3) along with appropriate vectors and plasmids, and allowed efficient production of the EGFR-TMD:JMD (residues 638-692) and HER2-TMD:JMD (residues 644-700).[115] Using the bacterial extracts the newly synthesised protein fragments tend to precipitate after the translation process, but the precipitate can be collected by

centrifugation, which also serves as the first step in purification. The precipitated fragments can be solubilised with detergents and purified by affinity chromatography.[115] The NMR measurements of the transmembrane segments embedded in the micelles show broader signals typical of oligomerisation, whereas the sharper signals from the juxtamembrane α-helical segment indicate interactions of this segment with both the lipid bilayer and water.[115] EGFR TMD fragments form dimers and NMR studies of these transmembrane segment interactions in the micelles revealed an inactive state in which a C-terminal GG4-like motif appears to be the driver of dimersation.[115] This motif is a sequence of two small side chain residues, with three other residues in between, and it is found at both ends of the helix in the transmembrane segments of all the catalytically active EGFR family members.[115,117,118] The inactive state occurring in micelles[115] contrasts with N-terminal GG4-like motifs observed in dimers thought to be active states captured in the lipid bicelles.[114] The structure observed in the micelles employs a less polar interface than the lipid bicelles, with most inter-monomeric contacts supplied by residues with bulky hydrophobic side chains.[115] These results highlight the importance of the characteristics of the phospholipid bilayer, specifically the polarisation properties and the access of the TM residues to water,[115] in determining the likely configuration of the transmembrane domain. Interestingly, in the micelles the EGFR-TMD elongates upon dimer formation,[115] with the generation of additional hydrogen bonds.[114,115,119] Additional NMR studies on an EGFR-TMD:JMD construct (residues 642-690)[116] reinforce this idea that the structure and lipid composition of the membrane influence the TMD structure.[116] The initial results with the cell-free synthesised EGFR-TMD-JMD protein indicated that the TMD was oligomerised in the micelle membrane and that the juxtamembrane domain folded into close proximity to the micelle membrane.

Bocharova and colleagues used their TMD:JMD construct (residues 642-690) in a range of membrane-like environments.[116] The C-terminal JMD was α-helical and membrane-bound in dodecylphosphocholine (DPC) micelles[116] but in bicelles of different sizes and compositions there were no α-helices.[116] In the DPC micelles two separate helices were seen, the TMD and the C-JMD, whereas in bicelles, only the TMD helix was formed.[116] These effects of membrane mimetics on the helicity and stability of the C-JMD raises the question as to which is a more biologically relevant model; is it the bicelles, in which a planar bilayer exists, with a relatively rigid surface, or the micelles, which have a looser (more accessible) surface?[116] In part this issue has been addressed by recent solid state NMR studies on another EGFR family member HER2.[120,121]

Developments in solid state NMR (ssNMR)[121] have improved membrane protein structural analysis. The TMD and JMD of HER2 have been studied by ssNMR, providing insights into how dimerisation of the TMD leads to changes in the membrane dynamics and activation of the HER2.[120,121] The ssNMR results emphasise the importance of the lipid environment in activation and signaling from receptor kinases.[120,121] Specifically, tight interactions were demonstrated between the negatively charged lipids and the JMD.[120] These tight interactions may have a role in maintaining the kinase domain in an inactive conformation:[122,123] when the TMD-JMD peptide contained the oncogenic V664E mutation within the TMD[120] a release of the JMD sequence from the negatively charged lipids was observed.[120] This oncogenic mutation has previously been shown to promote dimerisation of the TMD.[124,125] More evidence for the importance of lipids in determining the aggregation state and conformation of the JMD comes from molecular dynamics simulations which have reported that the JMDs of Receptor Tyrosine Kinases (RTKs) are key in promoting the clustering of anionic lipids, specifically phosphatidylserine and

PIP_2, in phospholipid bilayers.[126] This characteristic is highly conserved across RTKs - interactions between the JMD and lipids appear to play a key role in RTK structure and function.[126]

Further evidence for effects of specific ligands by and on the EGFR JMD have emerged[127] and the results from both the experimental analyses and the *in silico* analysis of dimeric JMD coiled-coil helical interactions demonstrate the presence of three different antiparallel coiled-coil states in the JMD.[127] In the first type of helical interaction, a hydrophobic interface mediated by leucine residues is formed between the two antiparallel helices of the JMDs (Figure 3A).[127] In the second JMD helical interaction class, an 'inside-out' version of this interaction occurs, i.e. an anti-parallel pair of helices, but the interface is polar. In this second type of interaction the two JMD helices are rotated 150 degrees in opposite directions around the helical axis and the hydrophobic leucine residues are moved to the outside (Figure 3B).[127] In a third conformation, an intermediate JMD helical interface is observed (Figure 3C).[127] The proportions of these structures depends on which growth factor is bound to the EGFR-ECD: the hydrophobic interface is observed upon binding with EGF or HB-EGF, whereas the polar interface is seen when TGF-α, AREG, EREG or epigen (EPGN) bind; the intermediate interface was seen when the EGFR was bound to BTC.[127] Each of these configurations correlates with differences in downstream signaling.[127] Previous work has shown that the seven growth factors which activate EGFR can have different effects on downstream signaling:[128-134] EGF, HB-EGF and BTC provide a shorter signaling pulse and greater receptor downregulation; TGF-α, AREG, EREG and EPGN promote receptor recycling and sustained signaling. These studies used a pro-fluorescent small molecule probe (an arsenical derivative of resorufin, RsAsH-EDT2)[135] and elegant tetracysteine derivatives in the JMD to show the three distinct patterns of helical coiled-coil interactions correlated with the different downstream signaling pathways, independent of endocytosis.[127]

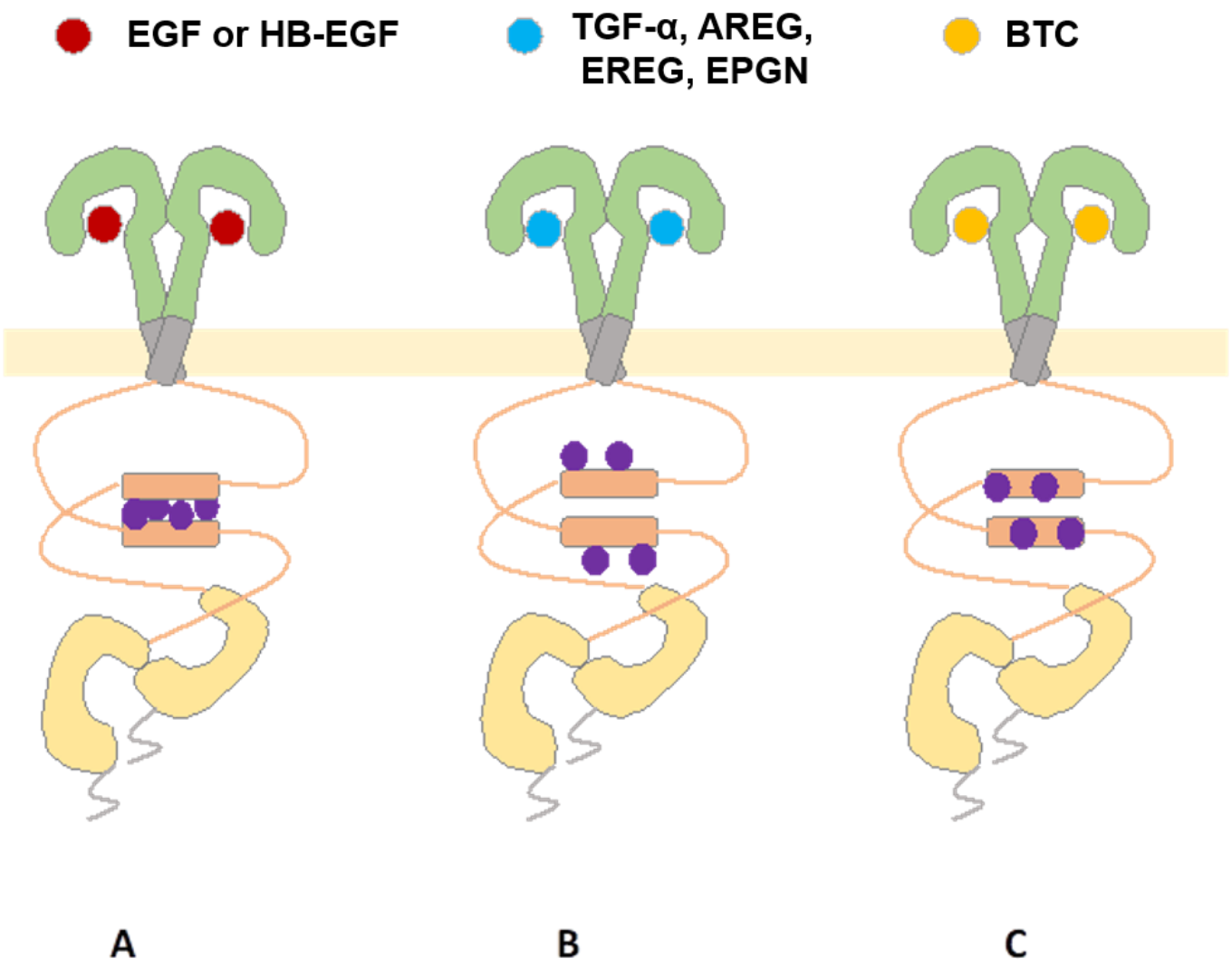


Figure 3. Helical interactions in the dimeric juxtamembrane domain (adapted from Doerner et al[127]). **A**, hydrophobic leucine residues interact with each other, **B**, the hydrophobic leucine residues project away from the interface of the two helices, leaving hydrophilic residues to dominate the interaction, and **C**, the leucine residues project sideways, forming an intermediate interface.

While the focus of EGFR targeting has traditionally been on either the ectodomain or the tyrosine kinase domain (TKD), the effects of peptides derived from the TMDs of EGFR and HER2 have been analysed along with an analogue of the C-terminal domains of the TMD or HER2.[136] A peptide, B2C-D, which incorporates the distal GG4-like motif of the TMD, appears to be active both *in vitro* and *in vivo*: inhibiting EGF induced EGFR phosphorylation and neuregulin (NRG) induced phosphorylation of HER3.[136] The viability of a range of human cancer cell lines was assayed after 72 hours of incubation with B2C-D, and while some cell lines were less affected, the proliferation of A431 cells was inhibited by 70% and in other cell lines (e.g. a pancreatic cancer cell line BXPC-3) there was 80% inhibition of *in vitro* proliferation.[136] An additional synergy was observed with gemcitabine in the pancreatic cancer cell line BXPC-3. This TMD peptide (B2C-D) is the first reported agent which can target TMDs in the HER family reducing EGFR signaling and slowing tumour growth.[136]

There are obvious limitations to investigations focussed on fragments of the EGFR which contain the JMD, the full-length receptor may well provide additional conformational restraints to the juxtamembrane region, reducing its flexibility as well as its access to water. There is a significant need for a clearer understanding of the conformation of the JMD in the context of full-length, membrane-bound receptor.[137]

5. The tyrosine kinase domain: structure, mutations, and targeting

The TKD (residues 688-979) is often mutated in cancerous tissue, e.g. L858R- and T790M-EGFR substitutions in NSCLC.[138-140] The structure of the EGFR-TKD, including 43 aa from the carboxyl-terminal tail, has been solved crystallographically, both free and with erlotinib bound.[141] These 3D-structures also reveal a putative intracellular dimerisation motif which is concentrated in the Leu^{955}-Val^{956}-Ile^{957}

segment lying between the TKD and the carboxyl-terminal tail, as well as clarifying the nature of the erlotinib binding site at atomic resolution.[141] The kinase domain of HER3 is not an active enzyme and therefore signaling from HER3 relies on the kinase activity of other EGFR family members.[142] Recent crystallographic work has described the structure of a heterodimer formed from the kinase domains of EGFR/HER3.[143] Further heterodimer structures of TKDs with HER3 carrying common cancer-associated mutations have also been solved; in each case the interactions between the kinases were found to enhance the allosteric potential of HER3.[143] The HER3 mutations – Q780R and E909G – stabilise the heterodimer interface of HER3 with EGFR, thereby enhancing downstream signaling initiated by the EGFR kinase.[143]

One oncogenic mutation in the hinge region of the EGFR-TKD, R776H, activates the kinase in a ligand independent manner.[144,145] This mutation is in the αC-β4 loop, which is the hinge point for inter-lobe and C-helix movement.[146] The mutation relieves auto-inhibitory interactions between the TKD C-helix and C-terminal tail.[147] The R776H mutation mediates activation in a manner dependent on the asymmetric dimer, or a higher order oligomer, which functions as a holoenzyme to phosphorylate monomeric EGFR. These results highlight the importance of the lateral phosphorylation pathway for EGFR oligomers. The lateral propagation of EGFR phosphorylation is an important component of receptor activation,[148] for instance it is possible to achieve global phosphorylation of EGFR in a single breast adenocarcinoma cell by local stimulation of EGFR with EGF.[149]

Mutations of the EGFR TKD often promote tumour progression.[138-140,150] It is known that EGFR mutations in the TKD are predictive of patient responses to tyrosine kinase inhibitors in lung cancer.[6,8,10] EGFR, HER2 and HER4 have been screened for TKD mutations in HER2 over-expressing breast cancer samples. Sequencing revealed several mutations including two in the EGFR, three in HER2 and three in

HER4.[151] None of the 11 patients with TKD mutations had even a partial response to HER2 targeted chemotherapy (trastuzumab), whereas 32% (21 of 65) of those patients with no TKD mutations (in EGFR, HER2 or HER 4) achieved a response to treatment.[151] The mutations occur in different parts of the TK domain so different activation mechanisms must drive the observed resistance to treatment.[151] *In silico* structural analysis was used to explore this resistance in more detail, and the effects of mutations in the adenosine triphosphate (ATP)/tyrosine kinase inhibitor (TKI) binding site were compared to those in the N-terminal and C-terminal lobes of the TKD.[151] Even within the ATP/TKI binding site, different mutations can have different effects: e.g., the EGFR N842I mutant is predicted to affect binding of TKI and ATP, whereas the EGFR G857E mutant appears to favour the active conformation (there are steric interactions which would disfavour the inactive conformation).[151]

Two of the most common oncogenic mutations in the EGFR-TKD, L858R[152,153] and T790M,[154,155] have been studied both as single and double mutations, and these mutants have been shown to shift the EGFR monomer towards active conformations.[156] During activation of the TKD it has been proposed that an asymmetric dimer is formed, with the C-lobe of the catalytic domain of the 'activator' interacting with the N-lobe of the 'receiver' TKD.[23,114,157] L858R stabilises the active kinase conformation, in which there is an extended confirmation of the activation loop and a hairpin in the N-terminal region, and strongly stabilises the α-C helix by the formation of a salt bridge between the positively charged R858 and negatively charged residues as 758, 761, and 762.[156] In contrast T790M favours activation by stabilising the hydrophobic cluster at the dimerisation interface.[156] Receptors bearing both the L858R and T790M mutations can be targeted using recently described covalent tyrosine kinase inhibitors.[158] The designs for these inhibitors were guided by molecular modelling.[158] These drugs are selective for the mutant EGFR and by targeting C797 in the ATP binding site with covalent bond formation, they

appear to overcome the effects of the molecular changes which can lead to resistance to conventional TKIs.[158] Using molecular dynamics and docking analyses, a double mutation in the TK region, G719S/T790M was analysed in presence of gefitinib, and it was found that the functional loop and activation loop move farther apart with the T790M mutation.[159] In contrast the G719S mutation caused ATP to move closer to the hinge region. The T790M mutation allowed the phosphorylated substrate to escape from the binding pocket more quickly.[159] In T790M mutated EGFR the distance between L718 and G796 is less than in WT EGFR and this results in a smaller slot in the hydrophobic region, which in turn facilitates exclusion of gefitinib from the binding pocket.[159] Structural studies are key to identifying the specific molecular changes in the enzyme that lead to resistance and will point the way to the drug modifications required to overcome resistance.[159]

Single nucleotide polymorphisms (SNPs) in EGFR-TKDs in cancer cells tend to lie closer to the catalytic cleft of the kinase.[160] Lung cancer-derived mutations in EGFR such as L858R can both induce oncogenic transformation by leading to constitutive kinase activity, but fortunately, this mutation also increases the sensitivity of the EGFR to specific inhibitors, such as gefitinib.[138,160] Grouping common SNPs into two categories, inherited disease-causing SNPs and cancer associated SNPs, and then mapping the SNPs to the catalytic core of protein kinases demonstrates very different distributions of SNPs in each category.[160] Whilst the common SNPs are randomly distributed over the whole catalytic domain, the cancer-associated SNPs occur more frequently in the P-loop (catalytic and nucleotide binding functions), the activation loop and catalytic loop.[160] These cancer-associated mutations also appear to stabilise the EGFR dimer.[160]

In unligated EGFR with mutations in the TKD, the ECD is more likely to be found in an extended conformation, i.e. similar to ligand-bound wildtype EGFR-ECD.[161] This supports the idea of oncogenic

signaling through oligomerisation of ligand-free, mutated EGFR driven by changes to the intracellular region of the EGFR which lead to changes in the conformation of the extracellular domain of the receptor, i.e. inside out signaling.[161]

Some oncogenic TKD mutations in NSCLC are known to confer hypersensitivity to erlotinib and gefitinib;[138,139,162] in part this may be due to metabolic stress induced by the inhibition of constitutively active oncogenic signaling, i.e. a reversal of the metabolic state induced by oncogene addiction.[163] In contrast, the T790M mutation of EGFR alters conformation of the ATP binding pocket,[164] thus reducing the effectiveness of erlotinib and gefitinib. EGFR-T790M has been the target of a high throughput drug screen, which identified a set of pyridones as potential therapeutics for patients with this mutation.[165] The leads from this screen were optimised to produce even more potent EGFR inhibitors (Ki ~4nM), which are highly selective (200x) for EGFR-T790M over the wild-type (WT)-EGFR.[165] In a similar approach, sub-nanomolar thienopyrimidine-based TKIs which inhibit the WT-EGFR and two of its oncogenic mutants: EGFR-L858R and EGFR-L861Q have been discovered.[166] Therapeutic targeting with small molecule TKIs, such as these new thienopyrimidine-based compounds, has several advantages, such as the selective inhibition of ligand-independent mutant EGFR, and potentially less side-effects than general EGFR inhibitors or mAbs antagonists.[166] As they can be much cheaper to produce small molecule drugs are likely to be more cost effective than mAbs.

The effects of most targeted therapies on EGFR dynamics and/or conformations during therapy are not known, but changes in inside-out signaling may explain the heterogeneous responses to therapies seen in cancer patients.[167] Coban and colleagues[167] used Forster resonance energy transfer/fluorescence imaging lifetime microscopy (FRET/FLIM) to shed light on the stability of EGFR dimers in the presence and absence of TKIs. Breast cancer cells (HCC1954) expressing EGFR were pre-treated with gefitinib,

then incubated with two-colour labelled EGF to assess protein-protein interactions with FRET/FLIM.[167] Gefitinib stabilised the ligand-bound EGFR homodimer in the HCC1954 cells.[167] Tyrosine phosphorylation of the EGFR intracellular domain (ICD) modulates EGFR dimerisation and/or oligomerisation.[167]

As described above specific activating mutations in the catalytic domain of EGFR in patients with NSCLC can convey an increased responsiveness to erlotinib and gefitinib,[138,139,162] whilst other mutations such as EGFR-T790M reduce the inhibitor effectiveness. Thus, the activating mutations can serve as predictive markers for response to TKIs.[168] The challenge of drug resistance, due to secondary mutations which occur after first-line treatment (for example T790M), is being met in part by structure-based design of new third-generation TKIs which covalently modify a reactive cysteine at the lip of the ATP binding cleft. Unfortunately, some of the warheads attached to the inhibitors are associated with high rates of cell efflux, so the killing effects are not as potent as expected from the Ki.[168] However, other rationally designed covalent EGFR-TKI drugs (e.g. AZD 9291) have favourable side-effect profiles in both animals and patients.[169] In the transgenic mouse model of lung cancer AZD 9291 induced a complete and sustained regression and in two patients AZD 9291 induced significant tumour regressions with minimal side-effects.[169]

Novel TKIs derived from pyrazolyl-thiazolinones have potent activity against EGFR and HER2 and have been studied using both quantitative structure-activity relationships (QSAR)[170] and *in silico* docking.[170] Refinements of QSAR techniques, such as utilisation of multiple EGFR TKD crystal structures to generate the docking results, appears to improve the performance of these models.[171] This contrasts with 3D-QSAR models,[172] in which a single protein structure is used to determine ligand conformations. Sun et al[171] used 19 protein structures for their ensemble-QSAR analysis: not only were they able to develop an

algorithm which can be used to predict the potency of new EGFR TKI, their results suggest new modifications of current inhibitors which are likely to be even more effective anti-cancer therapeutics.[171] Another novel approach exploited the Asp-Phe-Gly (DFG) motif in the activation loop of the EGFR-TKD as a target for structure-based drug design.[173] Subsequent synthesis and testing of these molecules confirmed that interactions between the inhibitor and this DFG motif could increase the inhibitor potency, whereas inhibitors with no charge interactions with this region had significantly reduced inhibitory activity.[173]

6. EGFR membrane dynamics

Despite progress in our understanding of the structural biology of the EGFR family, our knowledge of the configuration of the EGFR family members on the cell surface is still incomplete. Recent single-molecule studies[174] have assisted in the investigation of membrane receptor oligomerisation: stepwise photobleaching,[175] FRET,[176] sub-diffraction localisation microscopy,[177] and co-tracking.[178] The distribution of aggregation states for a receptor can now be interrogated at the molecular level without having to average multiple protein ensembles. Consequently different states of receptor complexes can be detected.[174] Photobleaching helps to determine the separation of molecules in the distance range of 10-50 nm, and has been used to show the organisation of EGFR at the nanometre scale.[179] In the 10-20nm range, FLImP (fluorophore localisation imaging with photobleaching) has been used to obtain improved resolution.[179,180] In the back-to-back EGFR dimer the ligand-to-ligand separation is 11nm.[36,37,179,180]

Confocal spectroscopy can quantify the level of EGFR oligomers and assess the aggregation state of the EGFR on the cell membrane. The aggregation behaviour of the EGFR including the time course of aggregation and responses to receptor inhibition and/or temperature changes was determined.[181,182] It is now clear that upon ligand stimulation the EGFR forms nanoscale aggregates on the cell surface. It has been demonstrated that antibody binding to EGFRs on the cell surface is significantly more stable than antibody binding to the isolated EGFR.[183] Protein-protein interaction kinetics on live cells can also be measured using LigandTracer[184,185] and Interaction Map[185,186] technologies.[183] The binding of radiolabelled ligands to EGFR has been measured over time and interestingly, the binding to the EGFR on different cells (A431 and HaCaT cells) produce different association constants and different Interaction Maps.[183] It has been suggested that the different ligand affinities could be due to sialation differences or the effects from the presence of autocrine ligands. The most compelling interpretation of the different interaction maps indicate that the HaCaT cells have a secondary form of the low affinity EGFR on their surface. It is curious that this form of the EGFR is not detectable on A431 cells. These results are a reminder of the need to consider the variability between individual cells and different cell lines which might contribute to these different results, including factors such as differing membrane morphologies, co-expression of EGFR mutants, extent of cross-talk with other EGFR family receptors, varying levels of autocrine TGF-α and/or other ligands and different levels of the enzymes which activate the precursor forms of the ligands.[187,188]

In the absence of an atom level resolution structure for the full length EGFR, other methods have been employed to gain insights into flexibility and conformational transitions associated with the full-length EGFR (FL-EGFR) structure, including molecular dynamics (MD) simulations.[189] The MD simulations of monomeric FL-EGFR in a lipid layer indicate that N-glycosylation is a critical determinant for the

equilibrium conformation of EGFR;[189] in particular for the orientation of the ectodomain relative to the plasma membrane (Figure 4).[189] The membrane contact sites for the ECD appear to be affected by the glycosylation, specifically, the glycan moieties serve as molecular cushions (I=N151, II=N172, III=N389 and IV = N420) which lift the ECD away from the membrane.[189] This elevation is most noted with regard to Domains I and III, which together form the ligand binding pocket.[189] Despite the glycosylation effects on the ECD, the TMD properties, JMD and the TKD of EGFR monomers in membranes appear relatively unaffected by N-glycosylation of the ECD.[189] Perhaps in the future these techniques can be applied to dimer and higher-level oligomer EGFR structures to determine the impacts of glycosylation and oligomerisation on the orientations of both the EGFR-ECD and EGFR-ICD with respect to the lipid bilayer.

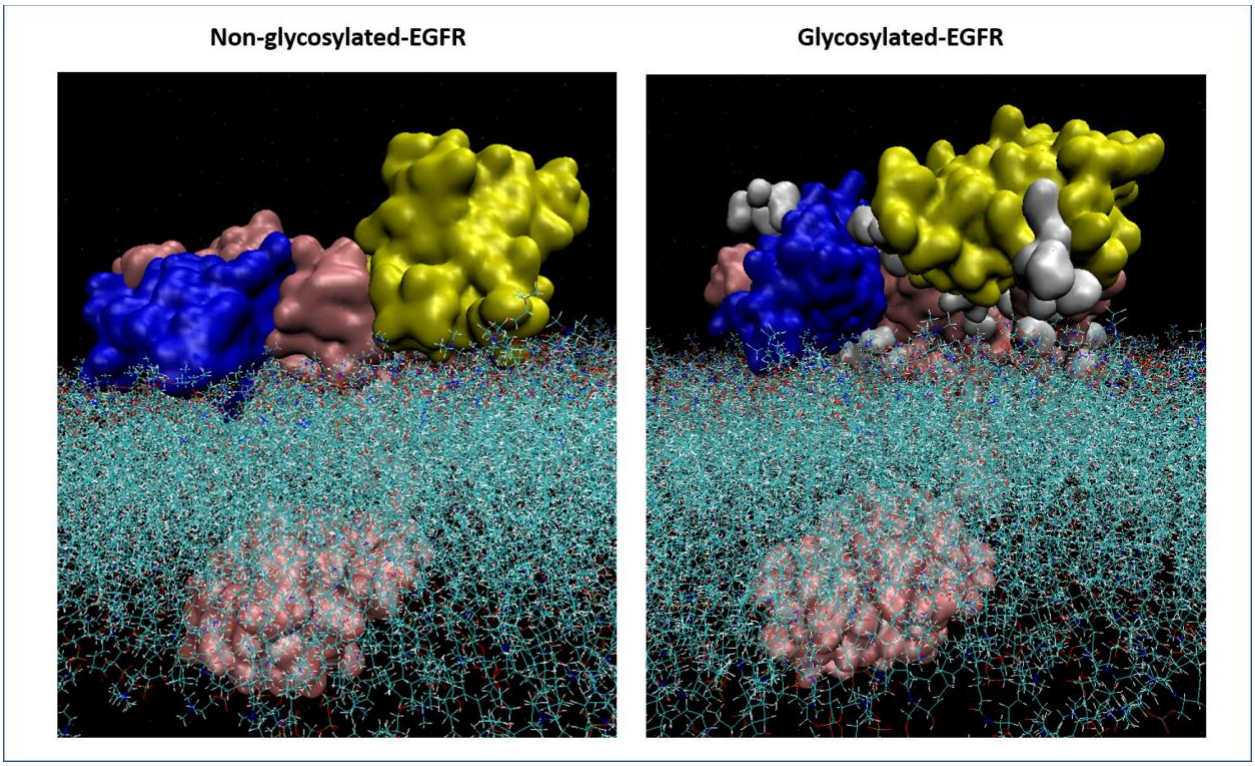


Figure 4. Molecular dynamics simulated structures of monomeric EGFR in the absence of ligand on

DOPC/SM/cholesterol membranes comparing glycosylated (**A**) with nonglycosylated (**B**) configurations, showing altered orientation of the ectodomain relative to the membrane with glycosylation. Domain I (blue), II (pink), III (yellow), IV and the intracellular kinase domain (pink). Domains I and III are lifted away from the membrane in the presence of glycans (grey/white) which appear to act as cushions, particularly affecting domains I and III, which together form the ligand binding pocket. In the absence of glycosylation, Domains I and III appear to move away from each other (adapted from supplementary data from Kaszuba et al[189] using VMD software,[24] http://www.ks.uiuc.edu/Research/vmd/ .

Paviolo and colleagues used gold nanoparticles (Au NPs), functionalised by conjugation to EGF, to bind to and interrogate the conformation and aggregation state of the full-length, cell-surface EGFR.[190] The Au NPs were made as nanorods (NR) or as spheres of different sizes.[190] Thiolated-EGF reacted with Au NPs (Figure 5) to functionalise the rods or the spheres.[190] The EGF-functionalised NRs activated EGFR when the EGFs on the NRs were separated by 40nm, but inhibited cell proliferation when the EGFs on the NRs were separated by more than 100nm; supporting the concept that the functional, aggregated EGFR had ligand binding sites with a separation in the range of 30-50nm and analysis of the effects of particle size and shape are likely to improve their use as anticancer therapeutics.[190] However, it should be noted that the thiolated EGF used for these studies may have been heterogeneous, i.e. the N-terminal amine group may not necessarily be the only amino group modified, e.g. the ε-amine(s) on lysine 28 and/or 48 could also have been converted to thiols. However, the modification of these lysines would most likely destroy the EGF binding, so only the α-amino modified-EGF is likely to produce functional EGF-NPs.

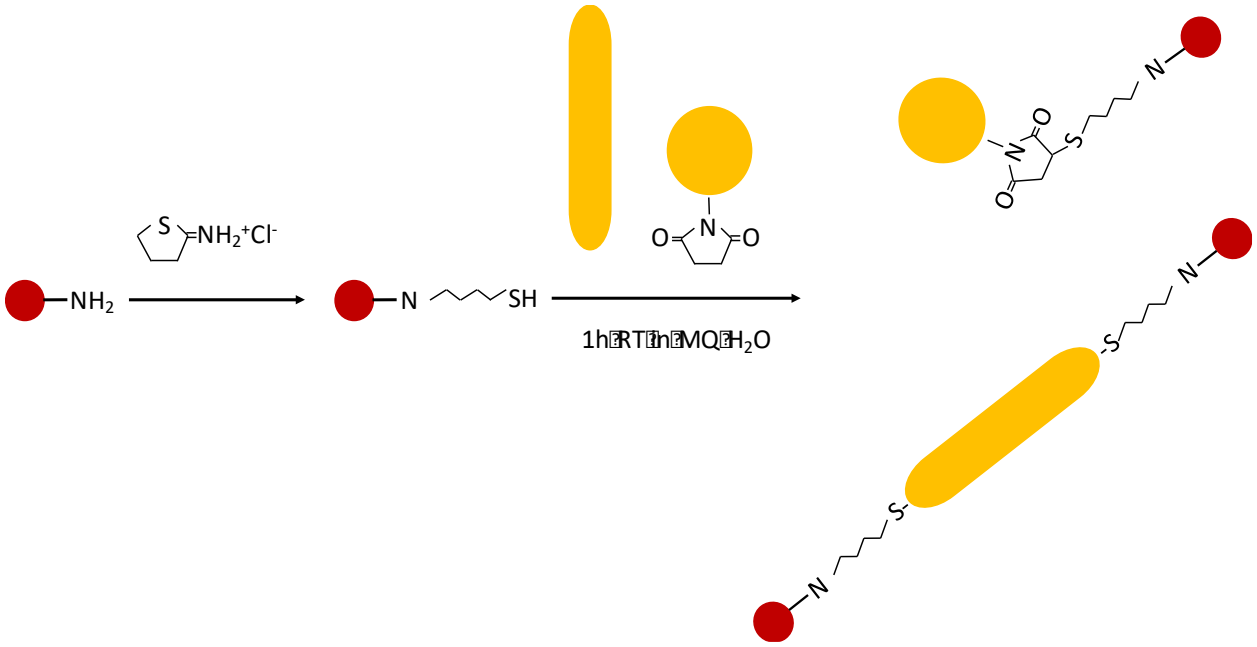


Figure 5. Reaction steps for the synthesis of EGF-conjugated gold nanoparticles, both rods and spheres (adapted from Paviolo et al[190]). The thiolation step was performed according to the procedure of Razumienko et al 2013[191] and on average only introduces one thiol per EGF.

7. EGFR hetero-oligomerisation and clustering

On the cell surface all four ERBB family members are capable of forming heterodimers,[192] but HER2 appears to be the preferred dimerisation partner.[192] Since HER2 has no ligand[193] and HER3 is an inactive kinase,[142] it is not surprising that only EGFR and HER4 appear to form homodimers/oligomers which contribute to downstream signaling.[192,194-196] It is likely that signaling by HER2/3 heterodimers involves the formation of higher-order oligomers.[194] Using luciferase fragment complementation imaging, the behaviour of five different HER receptor pairings (EGFR/EGFR, EGFR/HER2, EGFR/HER3, HER2/HER3, and HER3/HER3) has been analysed in detail.[194] It was clear from these results that when stimulated by EGF, the EGFR interacts with itself, HER2 and the HER3 in a hierarchical fashion, with the activation of

EGFR/EGFR ~ EGFR/HER2 >> EGFR/HER3.[194] These results also provide evidence for HER3/HER3 clustering (homodimers and higher-order oligomers), but the clusters appear to dissociate at very low concentrations of the ligand NRG-1β, possibly by stimulating the HER3 monomers to interact with other receptor family members.[194] NRG-1β induces an increase in luciferase complementation in both the EGFR/HER3 pairing, and the HER2/HER3 pairing.[194] The HER2/HER3 luciferase complementation is stimulated by low concentrations of NRG-1β (EC_{50} 0.38nM), a higher concentration of NRG-1β (EC_{50} 2nM) is required to induce luciferase expression in the EGFR/HER3 complex, which suggests the HER2/HER3 is a preferred pairing, so the overall stability when stimulated by NRG-1β is HER2/HER3 > EGFR/HER3 > HER3/HER3.[194] It is interesting to note that when EGF is used as the ligand there is a preference for the formation of EGFR homodimers and oligomers and the luciferase complementation signal from the EGFR/HER3 complex decreases.[194]

New insights into the nature of the interaction between EGFR and HER2 have been provided by long-timescale molecular dynamics simulations in which a single ligand is shown to be sufficient to stabilise the ectodomain interface to HER2 in heterodimers.[197] HER2 does not bind ligands and does not homodimerise, but as described in the previous paragraph HER2 heterodimerises with EGFR and/or HER3.[196,197] These molecular dynamics simulations predict that EGFR-HER2 dimers are stable for as long as the EGFR is bound to a ligand.[197] Ligand-bound EGFR-HER2 remains stable, as the ligand wedged between domains I and III of EGFR reduced the bending of domain II. In the absence of ligand, however, a large gap opens up between EGFR and HER2 in the heterodimer, specifically between the N-terminal portions of the domain IIs: in the absence of ligand domain II of EGFR bends away from the dimer interface (see Figure 6).[197]

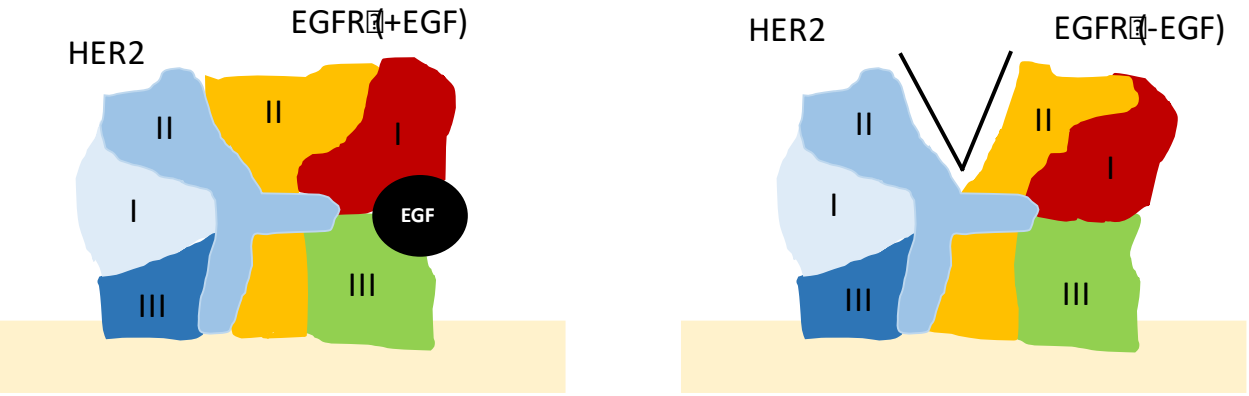


Figure 6. Molecular dynamics simulated structure of the EGFR-HER2 heterodimer with EGF (**A**) and without EGF (**B**) bound to EGFR. The gap in the dimer interface in the absence of EGF in denoted by the V shape (adapted from Arkhipov et al[197]). Light blue: HER2 Domain I. Medium blue: HER2 Domain II. Dark blue: HER2 Domain III. Red: EGFR Domain I. Yellow: EGFR Domain II. Green: EGFR Domain III. Black: EGF.

Head-to-tail bivalent ligands have been engineered with two EGF or two NRG moieties.[198,199] The linker between the two ligands was varied to produce inter-ligand distances of 5 or 18nm. The longer linkers should allow the two ligands to bind sites on adjacent back to back receptors and thereby drive receptor activation.[198] The bivalent EGF-EGF ligands, where the linker was >9nm, reduced EGFR/HER2 cell motility, as assessed by fluorescently labelling cells on collagen I- or fibronectin- coated surfaces, these bivalent ligands with the longer linkers did not stimulate cell survival or proliferation.[198] Sanchez-Palacios suggests that the longer linker EGF-EGF dimers sequestered the EGFR, but couldn't interact with the EGFR-HER2 heterodimer.[198] While several lines of evidence point to the existence of non-activated dimers, or predimers,[200-202] the conformations of the EGFR-ECD on membranes have not yet been characterised crystallographically or by high resolution cryo-electron microscopy.[203] It will be interesting to determine whether the bivalent ligands stimulate individual dimers or head-to-head

dimers in oligomer clusters. The concept of probing the EGFR family dynamics with bivalent ligands with different span lengths holds considerable promise,[198] but more extensive biophysical measurements are required before we can conclude that the signaling activities of homodimers, heterodimers or oligomers are influenced by the orientation of the ligand bridge.

Using nanobodies which only bind to ligand-free EGFR[34,204] where there is a tether between domains II and IV of the ECD it has been possible to demonstrate that the tethered form of the EGFR-ECD is involved in unligated EGFR/HER2 dimers.[203] Nevoltris and colleagues[203] generated two lama antibodies (D10 and E10) i.e. single chain antibodies, recognising the tethered form of the EGFR-ECD, and another (G10) recognising the untethered (extended) form of the EGFR-ECD. By labelling HER2 with a fluorescent probe and measuring FRET on unstimulated cells, a signal was detected when the EGFR was fluorescently labelled with the D10 and E10 antibodies, but not the fluorescently labelled G10 antibody.[203] When EGF was added the FRET signal decreased for the D10 and E10 antibodies, but increased for the G10 antibody.[203] The results provide clear evidence for preformed unligated EGFR:HER2 dimers where the EGFR is in the tethered conformation, upon ligand binding the EGFR in the EGFR:HER2 dimer transitions to the untethered conformer.[203]

The effects of membrane composition, co-expressed proteins and cellular dynamics combine to confound the analysis of the pre-formed EGFR complexes. However, the influence of different membrane environments in multiple cell lines, at several temperatures, on the localisation and levels of pre-formed EGFR dimers and higher order oligomers has been investigated by Yavas and colleagues.[205] The cell line, the receptor level and the location of the receptor with respect to the periphery or centre of the cell changes the level of EGFR dimerisation significantly. Cells with high levels of endogenous EGFR have more dimerisation, but there is a significant cell-to-cell variation even within the same cell

line.[205] Many cells show low or no fluorescence cross-correlation for the EGFR (using FCCS, i.e. there are very few dimers or higher order oligomers in these cells), other cells have much higher levels of an FCCS signal. On average almost two-thirds of the cell surface EGFR was present as pre-formed dimers or higher-order aggregates. Using dual-colour imaging total internal reflection fluorescence and cross-correlation spectroscopy (DC-ITIR-FCCS), the same group mapped the dimerisation of the human EGFR on the membranes of CHO-K1 cells.[205] They detected significant proportions of EGFR in the form of dimers, but again there was a large variation between individual cells.[205]

FRET measurements on live CHO cells in the absence of EGF have reported that the EGFR exists mainly as monomers, with only 10% as predimers.[20] After stimulation with EGF for 20s, 70% of the EGFR formed dimers, this dimerisation proceeded the increase in auto-phosphorylation, which plateaued at 90s post stimulation.[20] However, when the EGFR complex lifetime is similar to the measurement time, the fraction of dimers is likely to be under-estimated.[20] These experiments also provide evidence that there is likely to be a conformational change in the dimer when it binds even a single ligand.[20] There appear to be further organisational events before the full activation of the kinase domain; either ligand stimulated oligomer formation and/or a ligand stimulated conformational change may be required to activate the intracellular TKD.

The role of higher-order EGFR oligomers in total signaling outputs has been difficult to determine, but a study which assessed kinetics of both clustering and phosphorylation, has led to the development of a rule-based model to explain the current results.[21] This model predicts that cyclic EGFR tetramers are likely to be the major phosphorylated species and that the activated EGFR could be a side-to-side tetramer composed of cyclic dimers.[21]

Clearly, ligand binding stimulates the clustering of EGFR on the cell surface and the level of EGFR tyrosine kinase catalysed auto-phosphorylation correlates with the level of EGFR clustering.[22] The role of EGFR clustering on downstream signaling, has been studied by manipulating the scale of lateral diffusion of the receptor.[22] Large scale EGFR clusters (i.e.>1μm^2) have lower levels of EGFR phosphorylation per ligand than smaller (i.e.< 1μm^2) clusters.[22] When EGF ligand is anchored onto laterally immobile surfaces, the resulting slowly diffusing or immobile receptor/ligand complexes have higher ratios of phosphorylated EGFR to EGF than mobile complexes observed when EGF ligand is anchored to laterally mobile surfaces, thereby enabling larger clusters to form.[22] Stabley and colleagues[22] also provide evidence that F-actin-EGFR oligomer interactions initially prevent the formation of larger less active EGFR clusters. Their work highlights the importance of receptor spatial organisation to the fine-tuning function of both short-term and long-term EGFR signaling events.

Stochastic optical reconstruction microscopy (STORM) has also been used to analyse the effects of specific ligands on EGFR clustering and signaling from heterodimers.[206] When EGFR and HER3 signaling was analysed on sets of NR6 cells, it was clear that EGF induced clustering and phosphorylation of HER3 in much the same way as it induces EGFR clustering and phosphorylation; however, when NRG-1 is used as the ligand HER3 is induced to form a heterodimer with the EGFR but no clustering is detected. NRG-1 still induces the phosphorylation of HER3. NRG-1 phosphorylation of HER2 was dependent on the clustering of HER2:HER3 heterodimers. Clearly, the clustering of EGFR family members is important for signaling, however, the mechanism and extent of the clustering is influenced by the structure of the ligand. The importance of the context of EGFR activation was highlighted recently by Liang and colleagues[207] who discovered that whilst chemically crosslinked cell surface EGFR dimers become phosphorylated and recruit the appropriate adapter proteins, in the absence of ligand these

phosphorylated EGFR dimers do not activate Ras, ERK or Akt. A ligand induced conformational change in the EGFR and/or higher-order oligomerisation are required for the propagation of EGFR signaling within the cell. It would be interesting to know if activated Src , which can modify the activity of the EGFR and its substrates,[208] could also stimulate signaling from the chemically cross-linked EGFR.

The clustering behaviour of EGFR has also been observed in both normal lung epithelial cells and lung cancer cells using ultra-resolution microscopy.[209] Analysis of the cells using direct Stochastic Optical Reconstruction Microscopy (dSTORM) indicates that both the size and the number of these EGFR clusters is increased in some cancer cells.[209] Wang et al[209] showed that the number of EGFR clusters is dependent on the presence of the phospholipid PIP2: if the levels of PIP2 are reduced the number of EGFR clusters are reduced. These results emphasise the importance of anionic phospholipids in EGFR signaling. From our earlier discussion, it is possible that some of this phospholipid influence occurs by changing the conformation of the JMD.

Is receptor clustering per se sufficient to activate the EGFR in cells which are not dividing? To explore this question, superparamagnetic iron oxide nanoparticles (SPIONs) were tagged to EGFR on A431 cells, using either a monoclonal anti-EGFR antibody or a streptavidin molecule targeting a chimeric EGFR.[210] A magnetic field was applied to induce clustering:[210] ligand independent activation of EGFR, as measured by transphosphorylation and downstream signaling, was induced.[210] Thus activation of EGFR can be triggered by ligand-independent molecular crowding, such as that induced by overexpression or magnetically forced clustering.[210]

8. EGFR signaling

Historically it has been thought that ligand-induced receptor dimerisation is the key component of EGFR signaling,[211] however work done on an EGFR orthologue, the *Caenorhabditis elegans* LET-23 which is constitutively dimeric[212] suggests EGFR may be regulated by ligand induced allosteric changes in pre-existing receptor dimers.[212] Stimulation of the LET-23 receptor with its ligand LIN-3, appears to respond without alteration in oligomerisation status.[212] When mutational analyses were performed on LET-23 the pre-formed dimers were found to be stabilised by the dimerisation arm as is the case with other EGFR extracellular dimers.[36,37,212] This work supports the concept that the EGFR may be regulated by allosteric conformational changes within an existing dimer,[213] in a manner similar to that seen in insulin receptor family members.[212] The roles of EGFR oligomerisation, subsequent to the conformational changes associated with the dimer, on the levels of kinase activation in the LET-23 system are still to be determined.

The complexity of the EGFR responses to ligand binding is illustrated by the hyper-activity of a low-affinity EGF analogue EGF-L47V.[214] In WT EGF the residue 47 is leucine, which is known to be important for both EGFR binding and mitogenic activity,[215] however when this is replaced by valine, even though the binding affinity is reduced 100-fold, its mitogenic potential is enhanced.[214] This seeming discrepancy between binding and mitogenic potential highlights the importance EGFR display and dynamics on the action of the ligand. Decreased receptor degradation in the presence of the EGF-L47V analogue (compared the WT-EGF) allows continuous ligand stimulation over a longer time period.[214] Ligand is not simply important for the initiation of EGFR signaling but the nature of the ligand also determines the kinetics of receptor degradation/recycling and therefore the length of time cells are stimulated.[214]

Different EGFR ligands can stimulate different downstream signaling effects.[216,217] The recruitment of a number of different signaling proteins to the EGFR has been monitored using luciferase fragmentation imaging,[216] which reveals different recruitment patterns depending on the ligand with which the EGFR is stimulated.[216] These findings are consistent with the previously discussed structural insights into different ligand-specific JMD configurations (see Figure 3).[127] It is important to measure different cell functions of the EGFR complexes (e.g. motility, morphology, cell-cell adhesion, cell-matrix adhesion, differentiation, survival and proliferation) with more than just EGF as the ligand. The original crystal structures of the ligand-bound EGFR-ECD[36,37] demonstrate only small differences in the binding between TGF-α and EGF, such as the salt bridge between TGF-α residue Glu27 and EGFR residue Arg125, which is not replicated in the EGF:EGFR complex;[218] however, the kinetics of EGFR recycling are significantly different.[219]

Once some of the C-terminal tyrosine residues distal to the EGFR-TKD are phosphorylated they become docking sites for signaling proteins such as Shc2[220] and Grb2.[221] These docking proteins can propagate and amplify downstream signaling from the activated EGFR.[222] Single molecule pulldowns reveal that an EGFR ligand-stimulated interaction between Shc2 and Grb2 reduces Grb2 availability to bind to the EGFR phospho-sites, leaving these sites available for phosphatases.[222] These results explain the significant decrease in EGFR phosphorylation after 30s of ligand binding, and after the initial burst of kinase activity.[222]

Of the seven ligands for EGFR, Epigen (EPGN) was the last to be characterised[68,223] and consequently much of its biology is still emerging.[224] Investigations of the biology of EPGN illustrate the tissue specificity and unique biochemistry associated with the different ligands. EPGN is upregulated in colon cancer.[33,223] In the ovulatory follicle EPGN is upregulated in response to hormonal stimuli such as

luteinising hormone.[68,225,226] Transgenic mice which overexpress EPGN have enlarged sebaceous glands, and the mammary gland has a changed histology.[68] Mice lacking EPGN do not appear to have growth or viability abnormalities.[68] Crystal structures of EGFR-ECD bound to EPGN, as well as those bound to EREG induce less stable structures than EGF or TGF-α.[227] In particular, a dimer of EGFR-ECD bound to EPGN shows marked asymmetry,[227] in contrast to the 2-fold symmetry seen in both EGF[37] and TGF-α[36] -bound EGFR-ECD dimers. Interestingly as mentioned earlier for the EGF-L47V analogue, this weakened dimerisation could lead to more sustained EGFR signaling, directing breast cancer cells to differentiate or mobilise instead of proliferating.[227] At present it is not clear whether the sustained signaling is dominated by a changed conformational state which mediates a change in kinase activity or whether there may be less lysosomal degradation of the low affinity-ligand-EGFR complexes and/or more recycling of unligated EGFR to the cell surface from the endocytic compartment (see next section).

New insights into the activation and regulation of EGFR signaling come from recent studies on adult *Drosophila* intestine.[57] The equilibrium between cell loss and production is maintained via inhibition of stem cell division by enterocytes through E-cadherin regulated mechanisms, which prevent secretion of EGF by repressing the transcription of the pro-EGF maturation factor *rhomboid*.[57] *Rhomboid* cleaves EGF precursors for secretion.[57] Once an enterocyte becomes apoptotic, the loss of E-cadherin promotes cell division by release of cadherin-associated β-catenin and p120-catenin which induce *rhomboid*, which then trigger the activation of the EGFR in surrounding stem cells (Figure 7).[57]

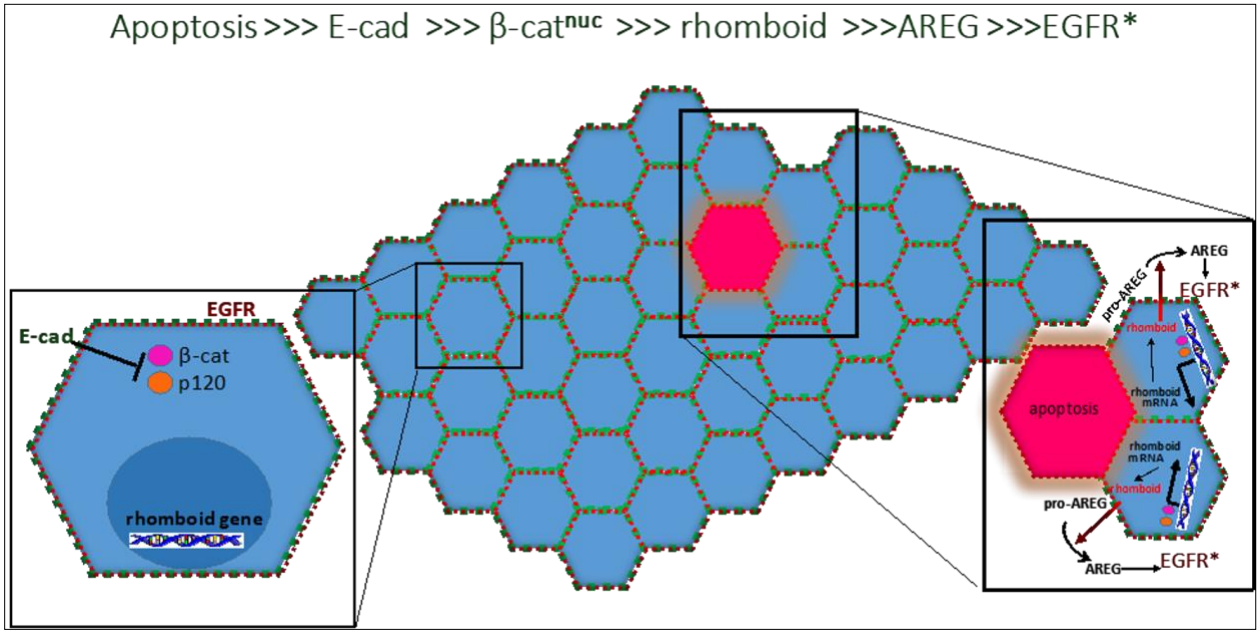
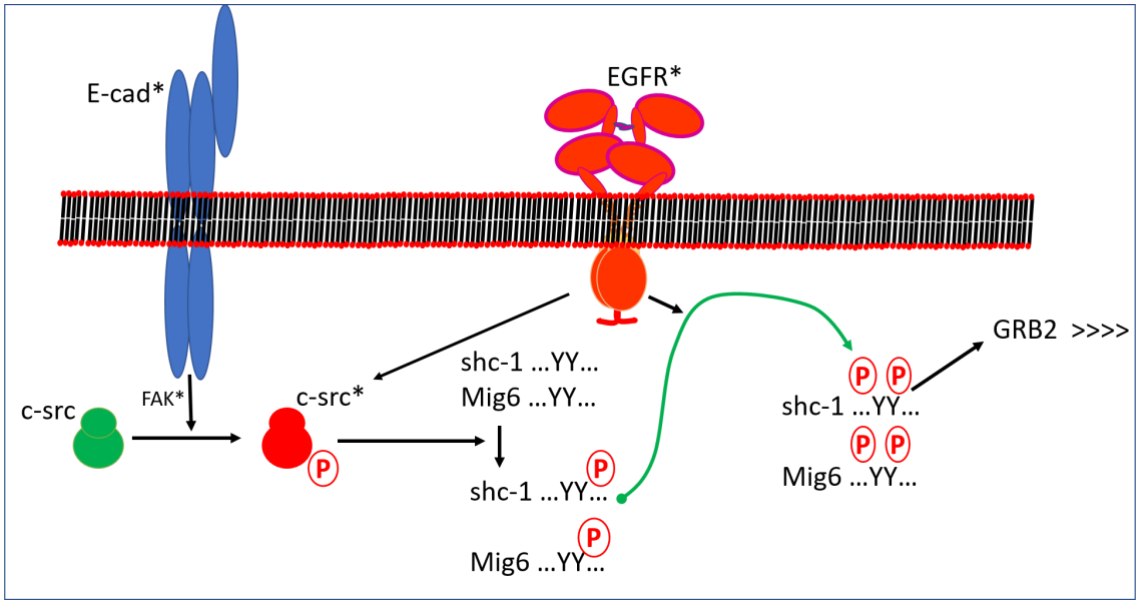


Figure 7.

Model for steady state equilibrium of enterocytes (adapted from Liang et al[57]). Quiescence is enforced by repression of enterocyte rho (expanded region on left). During apoptosis (pink enterocyte), induction of rho triggers local divisions (expanded region on right).

Until recently, little has been known about how the EGFR kinase specifically recognises its substrates, but two independent groups have now discovered a new consensus motif (YY) which is phosphorylated



by the EGFR kinase on the first tyrosine, only after the second tyrosine has been primed by Src-mediated phosphorylation.[208,228]

Figure 8 Ligand dependent activation of EGFR signaling initiates EGFR kinase activation of the >>> ras >>>Raf >>> ERK /2 and >>> PI3K >>> Akt >>>…. mTor pathways. However, the affinity of EGFR substrates is influenced by priming through enzymes such as c-Src* (where the * represents the activated state).[185,186] c-Src* priming of shc-1 increases phosphorylation by EGFR* allowing the recruitment of Grb-2 and initiation of the signaling pathway. c-Src can be activated by many signaling systems including E-cadherin associated, active cell-cell junctions, GPCR*, integrins*, ligand activated cytokine receptors via site specific tyrosine phosphorylation or phosphatases which remove the phosphate from the c-Src inhibitory site. EGFR* can also phosphorylate and activate c-Src,[229] thus amplifying its action on ERK signaling.

The specificity and affinity for binding of docking proteins to the tandem YY motif is increased by the priming phosphorylation of the second tyrosine.[208,228] In the Shc1 peptide this YY motif sits within a priming pocket where hydrogen-bonds are formed with K879 and A920 residues of the TKD of EGFR L858R.[208] This precise configuration is also seen when the Mig6 inhibitory peptide is bound to EGFR L858R.[208,228]

9. EGFR Endocytosis and Recycling

Inactive receptors are internalised spontaneously, but slowly and these unligated receptors are recycled rapidly back to the cell surface[230]. However, upon ligand binding, active receptors travel through the endosomal system where signaling continues and receptors are either recycled back to the cell surface or taken up into proteolytic lysosomes[231-233]. Several process including the phosphorylation of the β2 subunit of AP-2[234,235], receptor ubiquitylation through the E3 ubiquitin ligase Cbl[236], the presence of phosphoinositides in endosomal compartments[237,238] and vesicle interaction with the endoplasmic reticulum[239] influence the speed and fate of receptor endocytosis and trafficking and subsequently the intensity of the signaling. The persistence of ligand stimulated EGFR activity is regulated both by endocytosis and proteasome degradation.[240,241] Although EGF stimulates EGFR internalisation and degradation via lysosomes, other degradation pathways are also associated with the regulation of EGFR. Ack1 (activated Cdc42-associated kinase 1) has been linked to regulation of EGFR trafficking.[242] After EGF stimulation, this non-receptor tyrosine kinase co-precipitates with EGFR and co-localises with atg16-L positive structures.[242] It is proposed that this co-association is due to the precipitation of autophagosome membranes, in particular the sequestrosome.[242] When Ack1 is knocked down, EGF stimulated EGFR trafficking to the lysosomes occurs, so it appears that Ack1 has a role in diverting activated EGFR into another degradative pathway.[242]

The lysosomal degradation of EGFR, which is so important for terminating EGF-stimulated signaling, requires EGFR to be sorted into multi-vesicular endosomes.[237] The endosomal transmembrane

oncoprotein LAPTM4B, which inhibits intraluminal sorting and lysosomal degradation, amplifies and consequentially increases the duration of EGFR signaling.[237]

The N-terminal GG4-like dimerisation motif in the TMD (discussed previously) of the EGFR appears to be involved in clustering-induced internalisation.[243] EGF-induced internalisation is dependent on the presence of both the TMD dimerisation motif and an active kinase domain.[243] There may be a negative feedback role for TMD-dimerisation: in the absence of the GG4 motif, the EGFR is not internalised at the same rate and consequently more sustained signaling is observed.[243] Internalisation by either clathrin-mediated or clathrin-independent endocytosis[244-248] results in loss of signaling upon transportation of the active EGFR-ligand complexes into lysosomes, where they are degraded.[249] When the EGFR is internalised but not degraded, it can be recycled to the surface and continue to signal,[250] so the relationship between internalisation and sustained signaling is not linear.

Using advanced fluorescence spatial correlation spectroscopy, it has been observed that the diffusion of the EGFR on the cell membrane is best represented by a confined model of lateral diffusion.[251] This result is consistent with the tendency for EGFR to localise around caveolin invaginations.[251] The caveolae localisation appears to reduce the rate of degradation of the EGFR[251] and again this can enhance signaling from the ligand activated receptor.

10. EGFR Crosstalk

EGFR signaling can lead to activation of other signaling systems and vice versa. Understanding these cross interactions are important for predicting the effects of regulators on tissue biology.

Earlier discussions in this review indicate that the different EGFR family members can interact directly forming multiple signaling systems: in the EGFR:HER2 both kinases are activated when ligands binds to the EGFR, similarly the EGFR:HER4 is a dual specificity receptor kinase. Although the HER2:HER3 dimers/hetero-oligomers have only one type of EGFR family kinase, i.e. HER2, the interactions of the adapter proteins with phospho-HER3 lead to a broader activation of the signaling pathways. However, much richer and even more complex cellular signaling occurs through cross talk between other cell surface receptors such as the cadherins, integrins, other RTKs, GPCRs, Wnt, Notch, TGF-β and Hippo. Other regulatory proteins such as Lrig1, Ang2 and tissue plasminogen activator have been reported to influence the activity of the EGFR, perhaps by direct binding, however, these direct interactions have been hard to prove, as indirect activation of EGF-like ligands can be difficult to distinguish from a direct receptor activation process. The cross-talk experiments often rely on the analysis of recombinant proteins in cell-free systems in the absence of membranes, using higher than physiological concentrations of the different components – which can confound the interpretation of the experiments. However, at the cellular level the transactivation of EGFR phosphorylation, down-regulation and signaling has been a focus of cell signaling from the very earliest RTK experiments. Without trying to be comprehensive, we introduce some interesting recent reports related to the cross talk between receptor systems. It is interesting to reflect on the roles of each signaling system in different cellular processes such as survival, proliferation, differentiation, location and differentiation. In some cases, it is difficult to know whether both signaling pathways are required or whether signaling from one of the pathways controls the process – e.g. proliferation is often thought to be driven by EGFR signaling, but the consequences of EGFR signaling and signaling from the Hippo-YAP pathway on cell proliferation are often difficult to unravel.

Many G-protein coupled receptors (GPCR) have been reported to transactivate the EGFR including lysophosphatidic acids receptors,[252] angiotensin receptors, endothelin receptors, chemokine receptors, aminergic receptors, bile acid receptors and calcium receptors.[73] Many of these interactions are driven by EGF-family ligand activation via the induction of metalloproteinase activity at the cell surface or in the extracellular space.[67] GPCR activation has been linked to Src kinase activation which can activate the matrix metalloproteinases (MMPs) involved in the activation of pro-EGF-family ligands. There are also direct effects of activated Src on the phosphorylation of the EGFR (at Y845 and Y1110)[253] and as mentioned earlier that Src primes EGFR substrates.[208]

So activation of GPCRs can lead to direct modulation of EGFR signaling. The GPCR also modify the actions of β-arrestin-2, a scaffold protein which can influence ligand-independent phosphorylation of the EGFR.[254] At present the actions of β-arrestin-2 on the transactivation of the EGFR via the CPCRs appear to be cell context dependent[255] – in some cells EGFR phosphorylation is increased, in other cells, e.g. in a prostate cancer cell line, β-arrestin-2 blocks CXCR7 induced EGFR tyrosine phosphorylation.

One practical consequence of the cross talk between Notch signaling and EGFR signaling appears to be the development of resistance in breast cancers to EGFR targeting therapeutics.[72] The inhibition of the EGFR with tyrosine kinase inhibitors can inhibit proliferation of breast cancer cells, but this inhibition can be overcome by Notch-1 induced phosphorylation of Akt - thus promoting cell survival signals, even in the absence of EGFR activation. This knowledge led to experiments to combine inhibitors of EGFR and Notch-1 signaling: the results indicate a strong synergistic killing of breast cancer cells with this drug combination.[256] There is a similar interaction when breast cancer cells become resistant to trastuzumab:[257] in many primary breast cancers, driven by amplification/activation of HER2, the level of ERK activity suppresses the transcription of Notch-1 and γ-secretase (which is required for the

production of NICD-1 and Notch signaling); when HER2 is inhibited by trastuzumab, the levels of Notch-1 and γ-secretase increase, with increased phosphorylation of Akt and survival signaling. Using breast cancer 3D-mammosphere cultures it has been reported that the Notch/HER2 inhibitor combination reduced both proliferation and differentiation of the breast cancer stem cells.[257]

Tumours are complex tissue systems – consisting of the epithelial tumour cells, cancer associated fibroblasts, endothelial cells, lymphoid and inflammatory cells. It has been reported that mutant (activated) EGFR signaling leads to an increase in the secretion of IL6, which then stimulates its own receptor system and consequentially, pSTAT3 associated increases in VEGF and tumour blood vessel formation[258,259]. Consequently, inhibition of the EGFR system can have profound effects on tumour cell survival as well as reducing tumour angiogenesis. The converse stimulations, i.e. increased IL6 levels associated with tumour inflammation can activate the JAK2/STAT3 pathway, increasing levels of IGF-1R and signaling which can overcome the effects of inhibitors targeting the EGFR.[75]

The significant consequences of cross talk between signaling pathways is illustrated by the extraordinary interactions between Wnt and EGFR signaling in embryo development, tissue homeostasis and cancer.[260] There is evidence that activation of Wnt signaling leads to the induction of the EGF-like ligands responsible for the autocrine stimulation of cancer cells.[261] Activation of the Wnt receptor (frizzled) phosphorylates dishevelled which is inhibited by naked-2, the protein required to deliver TGF-α to the basolateral membrane of polarised cells.[44] EGFR activation can alter the phosphorylation and location of β-catenin. Depending on the tissue and its pathology, this EGFR perturbation of Lgr-5 and β-catenin function can antagonise or synergise with Wnt signaling.[262] In colon cancer and adenoma cells the interactions between Wnt and EGFR signaling control the formation of new crypts. Whilst the interactions between the Wnt and EGFR signaling pathways in normal intestinal cells is still being

unravelled, it is clear that both Wnt and EGFR signaling are required for the formation of new crypts *in vitro.*[263] Wnt, EGFR and Notch signaling converge to regulate GSK3β[264,265] and models have been developed to predict some of the cell differentiation outcomes associated with the combined actions of these pathways on muscle development.[266]

This section on cross talk would not be complete without a mention of the interactions between EGFR and TGF-β signaling. Both signaling systems are complex, but the cross talk is also a significant contributor to normal and tumour cell biology.[267] From its very first "appearance," TGF-β was associated with tumour biology and EGF signaling. Todaro and De Larco were investigating the factors secreted by mouse 3T3 fibroblasts transformed by the Moloney murine sarcoma virus.[268] The conditioned medium contained growth factors which competed for the binding of EGF to its receptor, stimulated normal fibroblast proliferation and the anchorage independent proliferation of cells. EGF could not account for all of these activities. They favoured the concept that the virally transformed cells produced sarcoma growth factors (SGFs), but it soon became apparent that SGFs were also present in normal tissue extracts and the two of the main components required to mimic the action of the SGFs were called TGF-α and TGF-β;[269] when both proteins were present the growth of cells in soft-agar was stimulated. TGF-α is closely related to EGF and stimulates the EGFR directly, but TGF-β defined a new protein family, with its own class of receptors and functional outcomes.[267] Three decades of research have revealed many of the intracellular reactions regulated by both proteins, but the pathways appeared almost completely independent. The complex tumour biology properties of the TGF-β family – an inhibitor of normal and pre-cancer cell proliferation (i.e. a tumour suppressor) and a stimulator of invasion and metastasis of advanced tumour cells (i.e. a tumour progressor) have captured the attention of cancer biologists for many years.[270] Recently it has become clear that TGF-β can stimulate the expression and activation of

the EGFR in advanced cancer cells and that this activation of the EGFR is required for the stimulation of invasion and metastasis by TGF-β.[271] The TGF-β induction of EGFR expression is enhanced in tumour cells where there is a defect in Smad4.[272] There are many more complexities in the interactions between EGFR and TGF-β signaling including the regulation of the ligand processing metalloproteinases[273] and interactions with both the Wnt and Hippo pathways through the control of YAP signaling.[274]

The pleiotropic roles of many growth factors and cytokines appear to be linked to the transactivation of the EGFR family, the proliferative changes associated with changes induced by the loss of E-cadherin signaling[57] and GPCR induced proliferation of cancer cells[67] are typical examples the potent effects of cross talk with the EGFR system. In the last few years the cross talk between EGFR signaling and Hippo/YAP signaling[274] have added an intriguing complexity with regard to the drivers of cell proliferation – are both ERK and YAP signals required, or can they act individually to regulate cell proliferation?

11. Overcoming resistance and new therapeutic strategies based on targeting the EGFR

As discussed earlier, secondary drug resistance in NSCLC can arise from EGFR-TKD mutations e.g. EGFR-T790M during TKI therapy,[164] however, there are intrinsic pathway modulations and feedback loops induced by TKIs which can also lead to drug resistance (Figure 9). One downstream effect of the action of EGFR kinase inhibitors is the reduction of Akt activity and a consequential reduction in Ets-1 activity.[275] Reduction in Ets-1 activity will reduce the expression of the DUSP6 phosphatase a negative regulator of ERK1/2. Thus EGFR-TKIs can lead to the indirect activation of ERK1/2.[275] Furthermore, in many lung cancers c-Src activity is elevated sufficiently to stimulate Ras GTP-loading and the activation

of Raf and MEK.[276] The combination of the TKI induced depletion of DUSP6 and the elevated c-Src leads to an activated Ras/MAPK pathway, increased survival and resistance to the TKIs.[276] More effective cell killing by the TKIs should be facilitated by combining the EGFR-TKIs with c-Src and/or MEK inhibitors.[276]

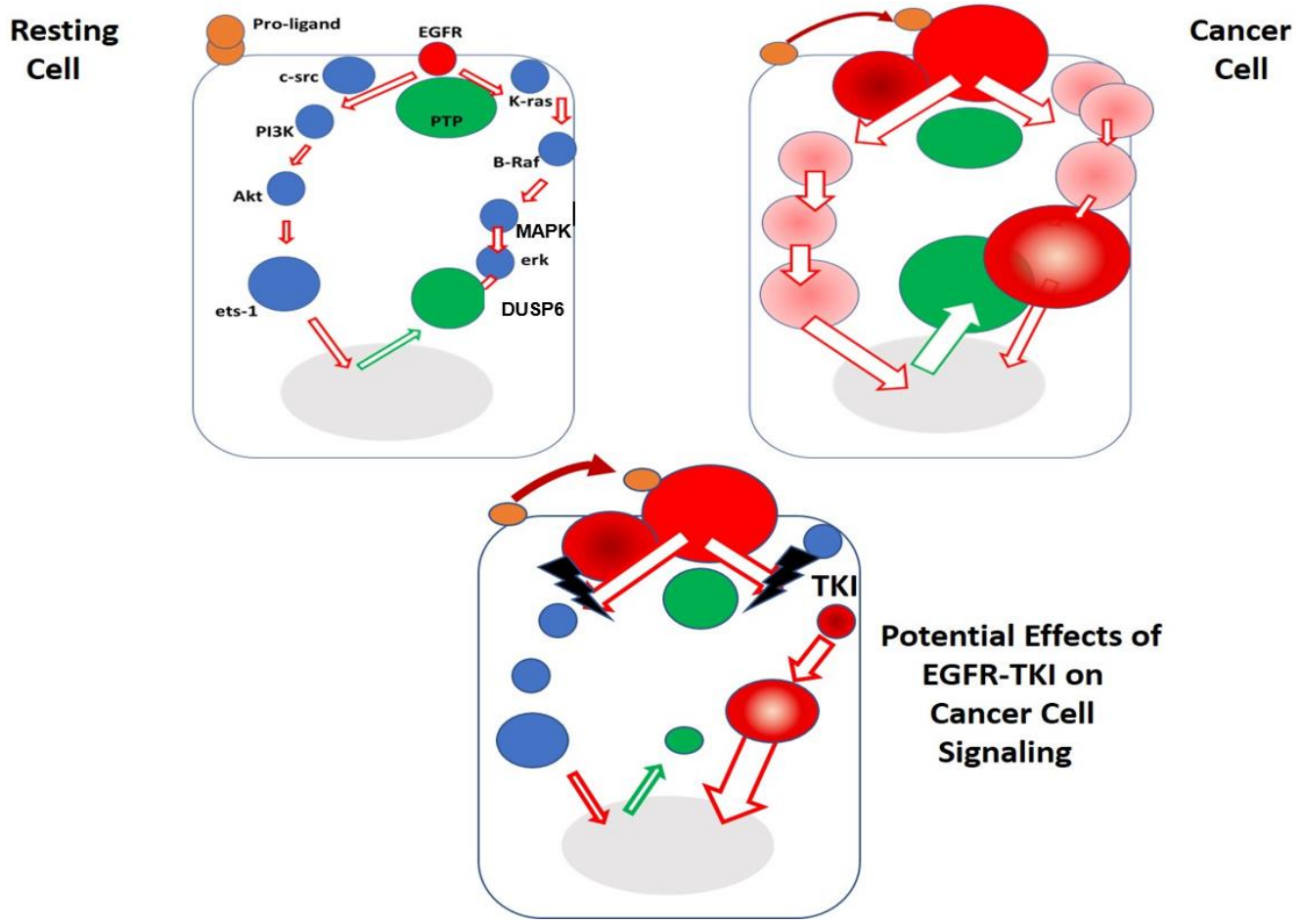


Figure 9 In resting cells signaling from the EGFR is controlled in part by ligand processing and the excess level of tyrosine phosphatases which prevent inappropriate signaling. Cancer cells have access to additional ligand (or the receptor is activated through mutation) and the MAPK/ERK pathway can be constitutively activated further by excess c-Src, oncogenic Kras or B-Raf. In many cancer cells ERK signaling is attenuated by the DUSP phosphatases. In attempting to kill cancer cells with EGFR-TKIs the initial EGFR substrate phosphorylations may be inhibited, but the stimulation of MAPK/ERK by c-Src, oncogenic Kras or B-Raf continues and since the EGFR kinase is inhibited PI3K activity decreases. The decreased PI3K also lowers the activity of Akt and the transcriptional activity of ets-1 which controls the

DUSP phosphatase (a negative regulator of ERK). Consequently, the use of the EGFR-TKI can lead to conditions which will actually stimulate cancer cell growth (see Phuchareon et al.[275]).

Another potential strategy to overcome acquired resistance to EGFR inhibition is the use of dual-target monoclonal antibodies which inhibit both EGFR and HER3,[16] e.g. the antibody MEHD7945A.[16] In cancer cells already resistant to cetuximab, erlotinib and/or radiation, MEDH7945A has been shown to inhibit tumour growth and cell-cycle progression, and in a head and neck squamous cell carcinoma tumour xenograft, MEDH7945A was more potent than either cetuximab or erlotinib alone.[16] *In vitro* MEDH7945A antibodies can also kill cells resistant to cetuximab and in combination with cisplatin there is extra cytotoxicity in some head and neck cancer cells lines.[277] The results from the clinical trials of MEDH7945A (commercial name Duligotuzumab) antibodies have been disappointing: in a head to head single agent comparison with cetuximab in head and neck cancer patients, MEDH7945A led to almost identical progression-free survival (4.2 vs. 4.0 months) and overall survival (7.2 vs. 8.7 months) data; the adverse events associated with MEDH7945A antibody treatment appeared to be higher than cetuximab.[278] In this study, MEDH7945A antibody treatment did not benefit head and neck cancer patients even when they had higher levels of neuregulin-1. Whilst accepting these results, other clinicians believe that MEDH7945A antibody treatment could still be a better option for patients who have not been treated with cetuximab.[279]

A potential single agent with dual targeting properties was discovered during computational screening to identify dual kinase/bromodomain inhibitors.[17] This dual EGFR-BRD4 inhibitor was synthesised and tested.[17] The BET bromodomain proteins such as B12D-4 are epigenetic readers recognising acetylated lysine residues on histones, and they regulate gene transcription.[280-282] This single molecule/dual

targeting approach may overcome the adaptive kinome reprogramming which can occur rapidly in cancer cells when they are treated with a single drug.[17] EGFR and BRD4 use orthogonal signaling networks, and different transcriptional regulation; it may be possible to achieve prolonged efficacy with a combination of compounds that target these two proteins or even with an inhibitor which targets both the EGFR family and the BET bromodomain.[17] In tumours such as glioma these targets appear to be critical drivers of proliferation.[280-282]

In cases of drug resistance, it is possible to use peptide analogues of EGF to inhibit the ligand-EGFR interaction.[18] The obvious limitation of this strategy is that multiple ligands bind to and activate EGFR, so the anti-EGF peptide antagonists need to be high affinity and long-lasting . Guardiola et al[18] made some inhibitory peptides but the affinities are in the mM range and too low to be effective ligand antagonists. Other studies attempting to interfere with the actions of EGFR ligands have used ligand traps with high affinity fragments of the EGFR-ECD or HER3-ECD to reduce autocrine ligand concentrations.[283,284] Whilst the results have met with some success *in vitro*, as yet the ligand traps have not been as potent as the anti-EGFR antibodies or the EGFR-TKIs *in vivo* .[283,284]

In optimising potential EGFR-based therapies for glioblastoma, both combination therapies and modifications of the delivery of therapeutic agents have been considered. One combination therapy study used convection enhanced delivery of nanocapsules loaded with EGFR-siRNA and anti-galectin-1-siRNA.[19] The continuous injection of the nanoparticles allowed diffusion and convection to occur simultaneously and minimises the systemic delivery of the agent.[285] The chitosan-lipid nanocapsules containing siRNAs targeting either EGFR or Galectin-1 or both were delivered to nude mice with established intracranial orthotopic U87MG glioblastomas.[19] This tumour system is particularly aggressive and without treatment all of the mice die within five weeks.[19] When the tumours had grown

too large there was no response to temozolomide alone, however, when temozolomide was used in combination with these two siRNAs delivered in the nanocapsules there was a significant improvement in survival of the mice.[19] While these results are promising, and the U87MG orthotopic tumour model is realistic in terms of invasiveness, tumour induced necrosis, and vascular alterations,[19] it should be noted that work on orthotopic brain tumours in nude mice does not always translate into clinical results relevant to the treatment of glioblastomas in humans.[286]

12. Conclusions and Future Directions

We have discussed recent developments in the structure and function of the EGFR family and their ligands. These discoveries are informing improved development of EGFR targeting drugs and even improving cancer treatment. There are new agents which can disrupt the formation of EGFR oligomers, and by combining EGFR targeting agents with complimentary epitopes, profound downregulation of ligand stimulated EGFR activity can be achieved. In the transmembrane and juxtamembrane domains of the EGFR, the presence of specific mutations and the lipid composition of the membrane both impact both structure and function of EGFR, underscoring the importance of these domains and the composition of the cell membrane on signaling from both normal and oncogenic forms of the EGFR. The TMD appears to have a key role in mediating specific intracellular responses to discrete ligands. The frequently mutated cytosolic tyrosine kinase domain is a particularly good example of an area where molecular modelling of mutated EGFR has led to the design of new inhibitors with more selectivity for oncogenic forms of the EGFR. This new generation of inhibitors could have many advantages, including potentially more favourable side-effect profiles compared to current mAbs or inhibitors. By using

specific inhibitors for the oncogenic variants of the EGFR, not only will there be less trauma for the patients, but the treatment is likely to be more cost effective.

When the EGFR is considered in the context of the membrane, although the experimental data is usually at lower resolution, molecular dynamic simulations have allowed for a new appreciation of structural variations caused by N-glycosylation, altering the contacts between the ECD and membrane lipids, with potential effects on the shape of the ligand-binding pocket.

While EGFR is implicated as a driver in some cancers, we have no current satisfactory anti-EGFR therapies. Anti-EGFR therapies are undermined by the development of resistance over time. The use of combination therapies in these patients is one possible approach to reducing resistance. Ligand trapping and ligand targeting have been explored, but have not yet been demonstrated to have adequate durability or potency to warrant clinical trials.

The majority of structural studies on the EGFR have been performed on fragments of the whole receptor, and while these are enlightening, many of the conformational restraints placed on the receptor when it is in its full-length, membrane bound form do not exist when shorter fragments are studied. The conformation of fragments is likely to be affected not only by the lack of interactions within monomeric EGFR, but also the interactions present in the dimers and higher-level oligomers. The *in silico* models of full-length EGFR embedded in membranes are restricted by the computational time and the effects of membrane lipid composition. When the EGFR family members are studied in cell lines, the high levels of variability in the behaviour of EGFR on individual cells is still confusing and we are not yet in a position to predict the EGFR responses or dynamics on different cell types.

Although the *in vitro* properties of many of the new inhibitors are promising, their application to the clinical context are often limited by the challenges of delivery to the tumour tissue (e.g. glioma patients) or the side effects associated with interactions with normal tissues. The limitations of murine orthotopic tumour models in glioblastoma have been mentioned, but it's worth noting that in terms of recapitulating the invasiveness, tumour induced necrosis, vascular alteration and immunological interactions of aggressive tumours, there are no perfect animal models for predicting the likely efficacy of drugs aimed at inhibiting EGFR family members.

The principle unanswered question in the structural biology of the EGFR is the lack of a full-length structure for a membrane bound EGFR. There is an urgent need for information that will allow us to explore the formation of dimers vs higher-level oligomers, the presence of absence of tethers, and the impact of ligand on the membrane bound structures of EGFR family complexes. Recent progress with electron microscopy on the insulin receptor embedded into nano-discs[287] indicates that high resolution structures for membrane associated EGFR family members are likely to be available soon. These structures will undoubtedly inform the design of more powerful and specific EGFR antagonists and inhibitors for treating cancer patients

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