MECHANISMS OF EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE ACTIVATION AND NUCLEAR TRAFFICKING

By

Monica Red Brewer

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

In partial fulfillment of the requirements for

the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

December, 2009

Nashville, Tennessee

Approved:

Professor Graham Carpenter

Professor Carlos Arteaga

Professor Bruce Carter

Professor Scott Hiebert

Professor Daniel Liebler

ACKNOWLEDGMENTS

First and foremost, I would like to thank my family for their unending support. The love and kindness they have bestowed upon me throughout my life has enabled me make achievements in my life that at times, I never thought were possible. To my parents, thank you for instilling in me the belief that I am capable of achieving anything if I have the motivation and work ethic. These basic principles have served me well, and will continue to do so throughout my life. To my brothers, I am so happy to have been blessed to have you both in my life and thank you for all of your support and advice. I am truly blessed to have married my best friend Bob, the love of my life, who has been there for me in good times and bad. Thank you Bob, for helping me through the trying times in my graduate career by lending your ear and your heart and helping me to laugh when I most needed it. I am fortunate to have known and loved Elizabeth Dalton Sleigh, who was an anchor that allowed me to always find my way home and to be in the right place at the right time. Thanks to all my extended family members: the McWhorters, Browns, Wisemans, Reds, Smiths, VanDusens, and Brewers.

This work would not have been possible without the guidance of my mentor, Dr. Graham Carpenter. I would like to thank him for allowing me the academic freedom to conceptualize my own ideas and experiments. For many graduate student mentors, it is a struggle to allow their students to try and fail. Often times, the mentor will define the exact course of the student. However, I

argue that by allowing me to fail along the way, in the end Graham has allowed me to succeed in becoming an independent thinker and scientist. This has been a hard-won and invaluable lesson and I am very grateful.

Finally, I would like to thank all the people that supported this endeavor throughout the years. Thanks to Dr. James Staros, Dr. Kristin Whitson, and Dr. Stefanie Whitson. Thanks to my thesis committee for your guidance, especially Dr. Scott Hiebert, who is always engaged and always asks the right questions. Thanks to Dr. Hong Jun Liao for all of your help – I'm convinced that you can clone anything. Thanks to Dr. Mark Lemmon for his collaboration and for taking the time to discuss not only our project, but principles of biophysics and proteins. Thanks to Ms. Marlene Jayne for your patience and advice.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

The epidermal growth factor (EGF) receptor, also known as ErbB-1, is a transmembrane tyrosine kinase that belongs to the eukaryotic protein kinase superfamily. The EGF receptor was first isolated in 1979 and cloned and sequenced in 1984 as the target of epidermal growth factor, which was isolated in 1960 by Stanley Cohen (Cohen 1960; Carpenter 1979; Ullrich, Coussens et al. 1984). Early studies showed EGF-dependent phenotypes in cell culture and in the mouse, which suggested an important role for the EGF receptor in development (Levi-Montalcini and Cohen 1956; Cohen 1962).

The EGF receptor has important functions not only in development, but also in pathologies such as cancer. Members of the ErbB family include ErbB-2, ErbB-3, and ErbB-4(Linggi and Carpenter 2006). Although highly homologous, the ErbBs do exhibit major differences in function. ErbB-2 is sometimes referred to as the 'orphan' receptor because it binds no known ligand and is the preferred dimerization partner for ErbB heterodimers (Klapper, Glathe et al. 1999). ErbB-3 contains an inactive kinase which is proposed to result from several nonconservative residue changes compared to ErbB-1(Guy, Platko et al. 1994). ErbB-4 undergoes sequential cleavage to produce a functional soluble kinase-active intracellular domain fragment (Ni, Murphy et al. 2001; Sardi, Murtie et al. 2006). Deletion of any of the *ErbB* receptor genes in murine transgenic models results in lethality at various developmental stages, and thus underscores the

requirement of these proteins in normal development. The EGF receptor is expressed in nearly all non-hematopoietic tissues and its activation results in a wide variety of cellular responses based on expression pattern and ligand availability (Riese and Stern 1998; Linggi and Carpenter 2006) Specifically, *EGF receptor*-deficient mice exhibit phenotypic defects in lung, gastrointestinal, skin, and eyelid development (Miettinen, Berger et al. 1995; Sibilia and Wagner 1995; Threadgill, Dlugosz et al. 1995). Sequence homology identified between the EGF receptor and its viral counterpart *v-erbB* in 1984, implicating the receptor in oncogenesis (Downward, Yarden et al. 1984). The role of mutant or aberrant EGF receptor expression in cancer has since been verified (Holbro, Civenni et al. 2003).

In this chapter I will review the current knowledge of EGF receptor structure and function.

EGF Receptor Structure

The epidermal growth factor receptor is the archetype for the structurally and functionally related ErbB family of receptor tyrosine kinases. The EGF receptor, also referred to as ErbB-1, is a Type I single-pass transmembrane glycoprotein and shares an architecture similar to ErbB-2, -3, and -4. The EGF receptor is initially synthesized as a transmembrane precursor polypeptide from which the 24 residue N-terminal signal sequence is cleaved (Ullrich, Coussens et al. 1984). Maturation of the receptor involves N-linked glycosylation of ten

consensus ectodomain asparagine residues within the endoplasmic reticulum. The mature 170kDa EGF receptor consists of a large ectodomain (70 kDa polypeptide, plus an additional ~35kDa in N-linked carbohydrate moieties) , composed of subdomains designated I-IV, a short 23 residue alpha-helical transmembrane domain (TM), and a 60kDa intracellular domain (ICD), which contains a cytosolic juxtamembrane (JM) region, tyrosine kinase domain (TKD), and a carboxyterminal region (CT)(Figure I).

The influence of each of these subdomains and regions on receptor dimerization and activation is reviewed in this chapter.

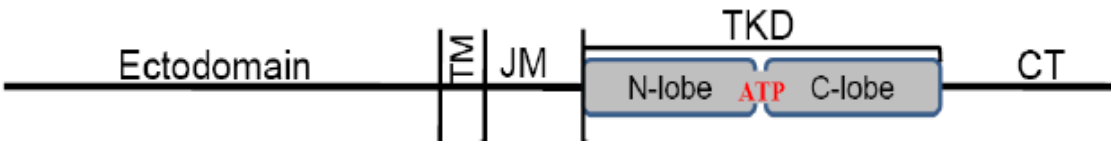


Figure 1. EGF Receptor Schematic. TM, transmembrane domain; JM juxtamembrane region; TKD, tyrosine kinase domain; CT, carboxyterminus.

Ectodomain

The ectodomain of ErbB-1 is composed of four subdomains and functions in dimerization of the receptor, a critical step required for tyrosine kinase activation. The mechanism for dimerization is mediated by ligand-binding to subdomains I and III of the ectodomain. Subdomains I and III are required for ligand binding, as evidenced by both cross-linking and chimeric chicken-human protein studies (Lax, Bellot et al. 1989; Woltjer, Lukas et al. 1992; Summerfield, Hudnall et al. 1996). These biochemical results have been confirmed by crystallographic data illustrating ligand-bound ErbB ectodomains (Garrett, McKern et al. 2002; Ogiso, Ishitani et al. 2002; Ferguson, Berger et al. 2003). Ligand-binding results in a conformational change in the ectodomain such that the 10 residue dimerization arm of subdomain II, a necessary component of receptor dimerization, is exposed to mediate contacts between ectodomain monomers. The dimerization arm is necessary, but not sufficient to drive receptor dimerization, as ligand is still required to induce dimer formation of extracellular domains (Elleman, Domagala et al. 2001; Garrett, McKern et al. 2002). However, mutational disruption of the dimerization arm results in increased dissociation of ligand as well as a decreased signaling capacity, implicating a role for the dimerization arm in the stabilization of the ligand-bound receptor (Mattoon, Klein et al. 2004).

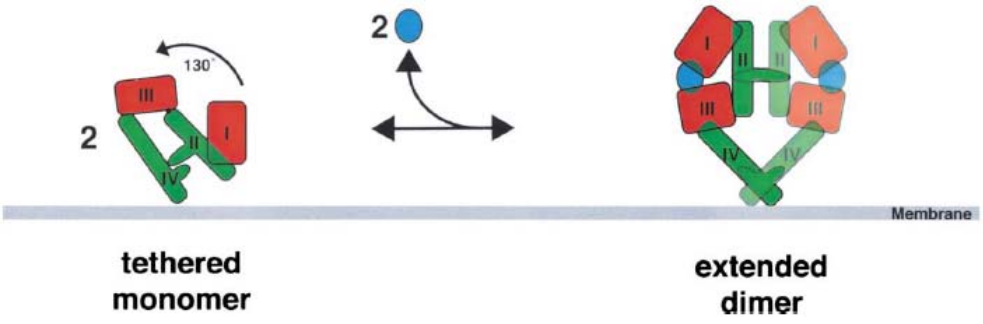
In addition to the role of ligands in dimerization of the receptor, the glycosylation state of specific asparagine residues in the ectodomain regulates ‘tethering’ of subdomains in the ‘closed’ conformation. The ‘tethered’, or closed

ectodomain conformation, is consistent with an unliganded inactive receptor state. The untethered, or extended ectodomain conformation, is brought about by ligand binding between subdomains I and III, resulting in a 130º movement of subdomain I as it 'swings' from the tethered to extended conformation and exposure of the dimerization arm (Figure 2).

The cysteine-rich subdomains II and IV have been traditionally thought to serve as intramolecular scaffolds (Abe, Odaka et al. 1998). Although subdomain II contains eight disulfide bonds, it not only contributes to the structural maintenance of the ectodomain, but also contains the aforementioned dimerization arm that is necessary for ligand-induced dimerization. Like subdomain II, subdomain IV also contributes to the organization of the ectodomain structure with seven disulfide bonds, but its deletion results in increased ligand affinity, suggesting a role in modulation of ligand-binding (Elleman, Domagala et al. 2001). Indeed, both crystallographic and biochemical studies have confirmed that this modulation is mediated by interdomain 'tethers' between subdomains II and IV (Cho and Leahy 2002; Ferguson, Berger et al. 2003).

Transmembrane Domain

The transmembrane domain is a 23 residue alpha helix. This domain is thought to play a regulatory role in the spatial orientation and association of ErbB receptors (Sharpe, Barber et al. 2002). This short sequence contains two GXXXG motifs originally described in the TM domain of glycoporin A (Figure 3).



Burgess et al. *Mol Cell* 12: 541-552 (2003)

Figure 2 . Schematic of EGF receptor Ectodomain Rearrangement upon Ligand-binding. Inactive ectodomain monomers are 'tethered' by glycosylation and intramolecular interactions. Upon ligand binding (blue oval) to subdomains I and III, conformational rearrangement of the ectodomain exposes the dimerization arm of subdomain II which then makes contacts with another ectodomain monomer in the extended conformation.

Two GXXXG motifs exist in the TM domains of all ErbBs, with the exception of ErbB-3. Mutational analysis demonstrates that these GXXXG motifs are required for interactions between TM domains of ErbB receptor monomers and are thought to facilitate dimerization (Mendrola, Berger et al. 2002). Mendrola et al. were able to discern a rank order for homodimerization of ErbB TM domains by employing a ToxR-chloramphenicol acetyltransferase (TOXCAT) assay. This assay employs the use of chimeras composed of the ErbB TM sequences and the transactivating sequences of the cholera ToxR protein which is only functional as a dimer. The TM domain of ErbB-4 was found to homodimerize most efficiently, while ErbB-1 and -2 were found to be equivalent in homodimerization capacity, and finally, the ErbB-3 TM domain homodimerized the least efficiently. This system was not used to assess the roles of the TM domains of ErbB family members in heterodimerization.

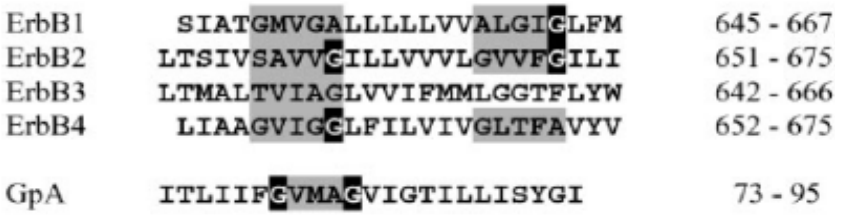
The mutation of Val664 to Glu in ErbB-2 from rat glioblastoma was reported to result in constitutive dimerization and activation of the receptor (Bargmann, Hung et al. 1986; Bargmann and Weinberg 1988). The equivalent 'Neu' mutation in human ErbB-2 (V659E) recapitulates the constitutive activation of ErbB-2 observed in rat glioblastoma (Segatto, King et al. 1988). Surprisingly, the 'Neu' mutation in human ErbB-2 results in a significant decrease in homodimerization as assessed by the TOXCAT assay, suggesting that the mutation drives activation not by enhancing dimer stability, but rather by altering the conformation of the receptor (Mendrola, Berger et al. 2002).

Tyrosine Kinase Domain

The tyrosine kinase domains of ErbB receptors are bi-lobal structures that contain elements conserved across the protein kinase superfamily. The major elements consist of a nucleotide-binding pocket, an activation loop, and an αC-helix (Hanks and Hunter 1995). The TKD is responsible for catalyzing hydrolysis of γ-phosphates of ATP for subsequent *trans*-phosphorylation of tyrosyl-containing substrates, including tyrosine residues located within the receptor carboxy terminus (Buhrow, Cohen et al. 1982; Cohen, Ushiro et al. 1982; Buhrow, Cohen et al. 1983; Downward, Waterfield et al. 1985; Honegger, Kris et al. 1989; Honegger, Schmidt et al. 1990). A functional tyrosine kinase is required to elicit EGF-dependent responses (Chen, Lazar et al. 1987).

Functional perturbation of catalysis, most commonly by mutation, can lead to kinase-dead or constitutively active phenotypes. Mutation of Lys721, yields a kinase negative receptor by preventing ATP binding within the nucleotide binding pocket of the receptor (Russo, Lukas et al. 1985). Mutation of Thr766 (Thr790 with signal sequence), commonly referred to as the 'gatekeeper' residue, to Met results in constitutive activation of the tyrosine kinase by increasing the affinity of the receptor for ATP (Kobayashi, Boggon et al. 2005; Yun, Boggon et al. 2007).

Structurally, the TKD is divided into a beta sheet-rich N-lobe and an alpha-helical-rich C-lobe. Until recently, the TKD was thought to dimerize in the same fashion as the ectodomain, as a symmetric dimer. However, research by the



Mendrola et al. JBC 277:4704-4712 (2002)

Figure 3. ErbB TM Domain Alignment. ErbB TM domain sequences are aligned with glycoporin A for comparison. GXXXG motifs are shaded gray and glycines at which substitutions result in significant reduction in dimerization are shaded black.

Kuriyan group in 2006, described an allosteric mode of activation in which TKDs form asymmetric dimers (Zhang, Gureasko et al. 2006). The precedent for asymmetric dimerization is supplied by the cyclin-Cylcin dependent kinase systems, in which a contact interface between opposing lobes of cyclin and cyclin dependent kinase monomers results in activation of the cyclins. For the EGF receptor, both crystallographic and biochemical data show that the C-lobe of a donor monomer contacts the N-lobe of an acceptor monomer to result in tyrosine kinase activation of the acceptor. Experimental mutations within the asymmetric TKD dimer interface of the intact EGF receptor demonstrate that this allosteric mechanism applies to the capacity of the full-length receptor to respond to EGF. This may explain how mutations in the ErbB-3 TKD activation interface contributes to an inactive ErbB3 kinase, but allows for activation of ErbB family members, namely ErbB2, by the ErbB3 kinase. In this case, ErbB-3 monomers may serve as “donor” monomers to activate ErbB-2 “acceptor” monomers.

Data suggests that the TKD-proximal juxtamembrane region and carboxy terminus serve regulatory roles in the allosteric mode of EGF receptor tyrosine kinase activation. Thiel and Carpenter (2007) demonstrate that the JM is required for kinase activation by showing loss of tyrosine phosphorylation of the EGF receptor upon deletion of the JM. The contribution of individual residues of the JM region to tyrosine kinase activation was not assessed in the study by Thiel and Carpenter (2007) and is the focus of research presented in Chapter III. Early studies from Welsh et al. (1991) demonstrated an increase in tyrosine kinase activity upon deletion of the carboxy terminus, indicating the presence of

an autoinhibitory domain. Jura et al. (2009) have identified residues of the EGF receptor carboxy terminus involved in mediating intramolecular autoinhibitory contacts. The role of receptor autophosphorylation in loss of autoinhibition has not been examined.

Carboxyterminus

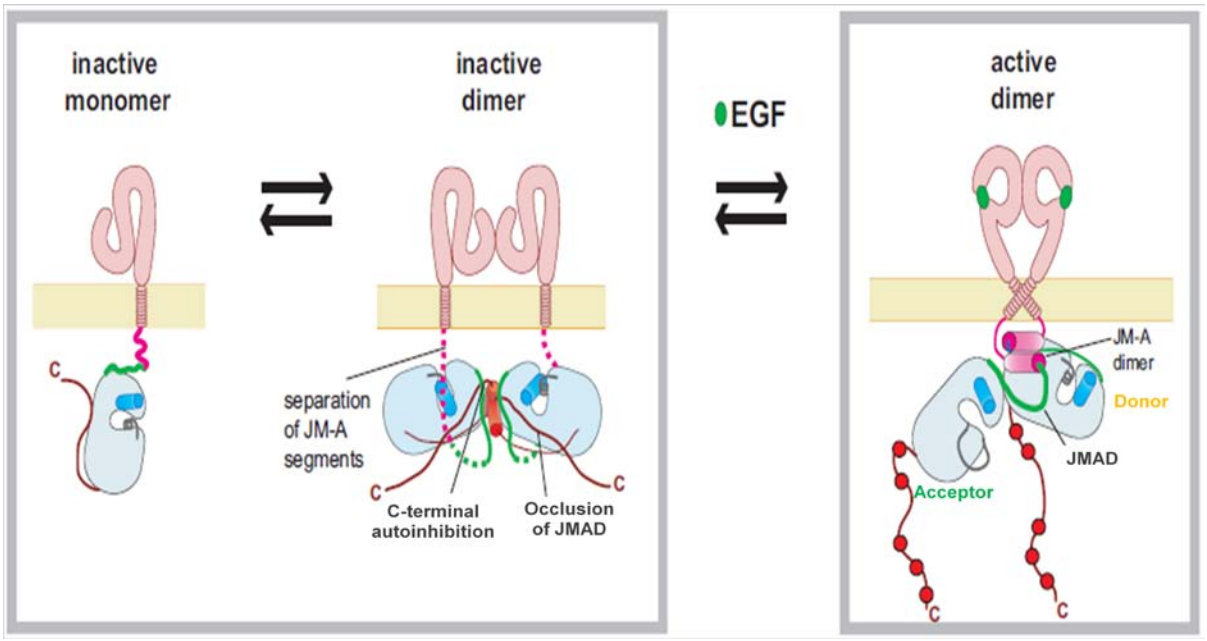
The carboxyterminus (CT) of the EGF receptor, spanning residues 996-1186, contains multiple sites of autophosphorylation as well as ubiquitination. Both of these post-translational modifications mediate effects on receptor signaling, as described below (*Ligand-Dependent EGF receptor Kinase Activation and Signaling*).

Besides the involvement of post-translational modifications in receptor behavior, truncation analysis showed that the CT mediates negative regulation of EGF receptor kinase activity (Walton, Chen et al. 1990). The structural basis for this CT-dependent negative regulation is now apparent. *Jura et al.* describe an autoinhibitory role for CT residues by illustrating intramolecular contacts of CT residues with C-lobe residues of the TKD via an inactive symmetric EGF receptor crystal structure (Jura, Endres et al. 2009). This inactive symmetric dimer is proposed to shift to the active asymmetric dimer state by increased local receptor concentration brought about by ligand-induced receptor oligomerization. The mechanism involves displacement of the intramolecular autoinhibitory CT

residues by the activating intermolecular juxtamembrane residues of a proximal kinase monomer (Figure 4).

Juxtamembrane Region

The 38 residue juxtamembrane (JM) region of the EGF receptor contains a variety of signaling and sorting motifs. A more extensive discussion of the juxtamembrane region, especially its role in EGF receptor activation, will be presented in Chapter 3 of this dissertation.



adapted from Jura et al. Cell (2009)

Figure 4. Role of Carboxy Terminal Residues in Autoinhibition of EGF Receptor Activation. The inactive symmetric dimer is maintained by intramolecular contacts of c-termial residues (red) with the N lobes of receptor monomers. Addition of EGF shifts the dimer equilibrium to the active asymmetric dimer, in which activating residues in the juxtamembrane region (green) occupy the same positions as the autoinhibitory c-terminal residues in the inactive state. JM-A (pink lines) denotes residues 645-663, also described as the exon-17 encoded portion of the JM, that immediately preceeds the Juxtamembrane Activation Domain (JMAD). The JM-A segments of the JM region are proposed to form antiparallel alpha helices in the active asymmetric dimer (pink cylinders, right panel).

EGF Receptor Function

Ligand-Dependent EGF Receptor Kinase Activation and Signaling

One of the most basic functions of transmembrane proteins is to mediate cellular responses to extracellular stimuli. Ligand-binding initiates EGF receptor responses. The seven known EGF receptor ligands are EGF, epigen, epiregulin, betacellulin, heparin-binding EGF, amphiregulin, and transforming growth factor α. Generation of mature diffusible ligands occurs by proteolysis of the transmembrane pro-forms at the cell surface (Schneider and Wolf 2009). Ligand-binding to EGF receptor results in the conformational rearrangement of the ectodomain of the receptor from a tethered to an untethered form, ultimately leading to its activation (Figure 2).

The receptor possesses tyrosine kinase activity whereby ATP-binding in the nucleotide-binding pocket of the intracellular tyrosine kinase domain is followed by hydrolysis of the terminal γ-phosphate of ATP and autophosphorylation of c-terminal tyrosine residues in *trans or* phosphorylation of exogenous substrate proteins, such as phospholipase-C γ1. The receptor contains seven known sites of tyrosine autophosphorylation that are contained in the carboxyterminus, as well as one Src phosphorylation site (Tyr845) located on the activation loop (Figure 4)(Biscardi, Maa et al. 1999). Tyrosine kinase activity of the receptor is separable from receptor autophosphorylation. In cells the CT-truncated receptor maintains its capacity to phosphorylate substrates. In *in vitro* kinase assays employing the purified TKD, exogenous substrates are efficiently

phosphorylated (Walton, Chen et al. 1990; Zhang, Gureasko et al. 2006). The function of tyrosine phosphorylation of the receptor is activation of downstream signaling pathways by recruitment of proteins containing either Src homology-2 (SH2) domains or phosphotyrosine binding (PTB) domains (Figure 5). For example, PLCγ-1 is recruited to the receptor by its SH2 domain, which associates with pTyr992. PLCγ-1 is then tyrosine phosphorylated and activated by the receptor kinase, resulting in hydrolysis of phosphatidyl inositol-4,5-bisphosphosphate to form the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. Another example is the recruitment of the adaptor protein Grb2 to pTyr1068. Grb2 then acts as an intermediary in the activation of the Ras/MAPK pathway. The PTB- and SH2-containing adaptor protein Shc can be recruited to EGF receptor phosphorylated at either Tyr1148 or Tyr1173. This propagation of signaling leads to cellular responses, such as proliferation and survival(Yarden and Sliwkowski 2001).

EGF receptor-propagated signaling pathways include the mitogen-activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) pathway, and signal transducer and activator of transcription (STAT) pathway. Aberrant upregulation of MAPK, PI3K/Akt, and STAT signaling pathways is a common feature of EGF receptor-dependent cancers.

The general mechanism of MAPK activation is described above, where recruitment of the constitutively associated Grb2-Sos complex to sites of tyrosine phosphorylation of the receptor initiates the MAPK signaling cascade.

The phosphoinositide 3-kinase pathway, sometimes referred to as the PI3K/Akt pathway, is initiated downstream of activated EGF receptor by the PLC-γ catalyzed generation of the second messengers phosphatidylinositol 4,5-bisphosphate (PIP2) and diacylglycerol (DAG). DAG then activates PI3K, which then directly activates Akt. Akt is known to have a variety of substrates which, when phosphorylated, result in cellular survival. Such Akt substrate proteins include mTOR and XIAP, which are known to be involved in protein synthesis and inhibition of apoptosis, respectively.

The signal transducer and activator of transcription (STAT) proteins are nucleo-cytoplasmic proteins that can be phosphorylated directly by the EGF receptor. Phosphorylated Stat proteins are dimeric and localized to the nuclear compartment where they mediate cellular survival and proliferation via transcriptional activation on gamma activated sites.

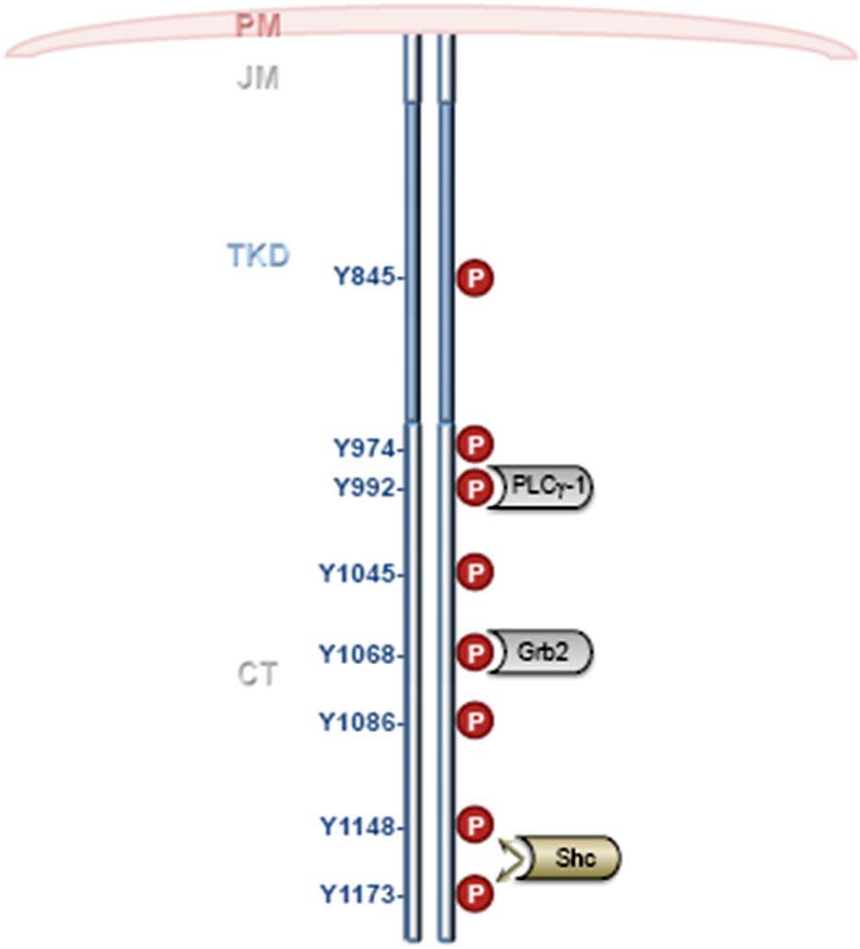


Figure 4. EGF Receptor Intracellular Domain. Autophosphorylation sites are indicated. The Src phosphorylation site Tyr 845 is also indicated. Examples of known SH2-containing proteins, PLCγ-1 and Grb2, and their docking sites are shown, as well as the PTB- and SH2-containing protein Shc.

Receptor Trafficking

Activated ErbB receptor dimers cluster into clathrin-coated pits, which leads to receptor internalization (Vieira, Lamaze et al. 1996). Receptor internalization has been shown to require the endocytic epsin proteins and the cargo recognition protein AP-2(Rappoport and Simon 2009). The AP-2 protein associates directly with the EGF receptor and data from Jura et al. (2009) suggest that specific carboxy terminal residues are responsible for this interaction. Association of activated receptor with epsin or epsin-15 via AP-2 results in localization within early endocytic vesicles. Ubiquitin moieties present on the EGF receptor after activation have also been demonstrated to mediate interaction with epsin proteins (Stang, Blystad et al. 2004; Kazazic, Bertelsen et al. 2009). Thus, data demonstrates a role for ubiquitin in the internalization of activated receptor populations.

Internalized receptors have different fates, which are dependent on cell type, receptor concentration, cytosolic factors, and post-translational modifications (Sorkin and Goh 2009). As depicted in Figure 5, the majority of internalized EGF receptor is subject to lysosomal degradation (Sorkin and Von Zastrow 2002). Receptor recycling is also observed, as internalized receptor is sorted back to the plasma membrane (Sorkin and Von Zastrow 2002).

Finally, and of particular importance in Chapter 4, activated, mature EGFR is trafficked to the mitochondria or nucleus (Lin, Makino et al. 2001; Boerner, Demory et al. 2004; Lo, Hsu et al. 2005). The mechanism by which mature intact

EGF receptor is trafficked to the nucleus is unknown. A more extensive discussion of nuclear trafficking of the EGF receptor will be presented in Chapter 4 of this dissertation.

EGF Receptor in Cancer

The EGF receptor has described roles in various cancers, such as head and neck, breast, brain, gastrointestinal, and lung cancers (Yarden and Sliwkowski 2001). The receptor mediates tumorigenesis by amplification and/or mutation. The tumorigenesis resulting from amplified receptor is due to the increased activation and proliferative signaling brought about by elevated receptor concentrations, as seen in glioblastoma (Yamazaki, Fukui et al. 1988; Zawrocki and Biernat 2005).

In addition to amplification, mutation of the receptor results in tumorigenesis. The most common deletion mutation (first identified in glioblastoma) is known as ErbB-1-VIII, in which exons 2-7 (that encodes a large portion of the ectodomain) are missing. This results in a constitutively active receptor (Wong, Ruppert et al. 1992; Frederick, Wang et al. 2000). In lung cancer, small deletions and point mutations were found in the tyrosine kinase domain of the EGF receptor that renders the receptor constitutively active (Sharma, Bell et al. 2007).

Both amplified wild-type receptors and mutant receptors are the focus of a variety of therapeutics. Two categories of therapeutics targeted towards ErbB-1

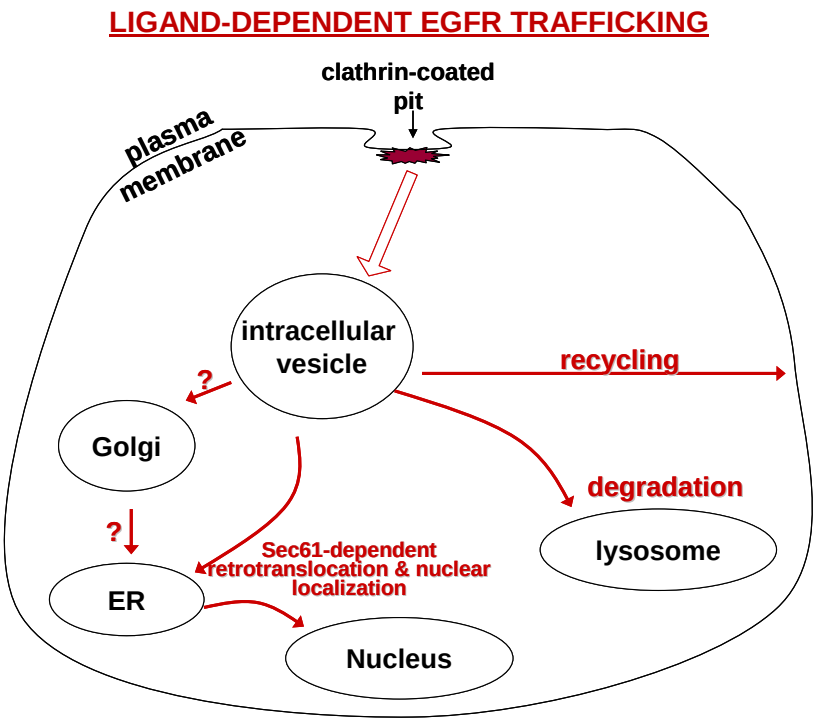


Figure 5. Ligand-dependent EGFR Trafficking Schematic. Major trafficking pathways of activated cell-surface EGFR are indicated and include recycling, degradation, and the novel ER-localization and Sec61-dependent nuclear localization.

are currently approved for clinical use and/or are in clinical trials. The first category of ErbB-1-directed therapeutics is humanized monoclonal antibodies directed to the ectodomain of the receptor, such as Erbitux (cetuximab/C-225, Bristol-Myers Squibb). This antibody is currently FDA approved for the treatment of both colorectal and head and neck cancers. Antibodies directed towards the ErbB-1 ectodomain function by preventing ligand-binding and dimerization of the receptor.

Small molecule inhibitors represent a second avenue of receptor inhibition. All currently available EGF receptor tyrosine kinase inhibitors (TKIs) are ATP-mimetics that compete with ATP for binding to the nucleotide-binding pocket. Two basic classes of TKIs are reversible and irreversible small molecules. Examples of the reversible TKIs include gefitinib (Iressa, Astra Zeneca) and erlotinib (Tarceva, Genentech). The irreversible inhibitors, such as HKI-272, form a covalent linkage with Cys727 in the ATP-binding pocket, thus preventing ATP-mediated autophosphorylation. Unfortunately, examination of patients indicates that while TKIs may be preliminarily efficacious, resulting in a temporary reduction of in the number and size of tumors, mutations frequently arise within the EGF receptor TKD that allow activation in the presence of small molecule inhibitors - a phenomenon known as acquired resistance. For this population of patients, progression of disease is observed. The most frequently occurring acquired resistance mutation in patients treated with EGF receptor-directed TKIs is T766M (Balak, Gong et al. 2006; Kosaka, Yatabe et al. 2006). Interestingly, the structurally equivalent residue in the Bcr-Abl protein, Thr 315 is

also frequently mutated upon treatment of chronic myelogenous leukemia with Bcr-Abl ATP-mimetics such as imatinib (Gleevec) (Shah, Nicoll et al. 2002).

EGF receptor nomenclature used in this dissertation will refer to the mature receptor sequence, that is, receptor from which the signal peptide has been cleaved, resulting in a minus 24 amino acid difference from the cDNA-derived sequence. For example, the gatekeeper mutation is referred to as either T766M (mature) or T790M (cDNA) in the literature.

Data presented in this dissertation is directed toward elucidating both the role of the juxtamembrane region in the allosteric activation of the EGF receptor and the role of the p97 AAA-ATPase in mediating ligand-dependent nuclear trafficking of the intact receptor. Chapter III is dedicated to the identification of juxtamembrane residues required for tyrosine kinase activation. Biochemical and biological experiments are presented that demonstrate the existence of a juxtamembrane activation domain that is required for receptor activation as well as novel patient mutations within this new domain that result in constitutive tyrosine kinase activation and tumorigenesis in a xenograft model. The focus of Chapter IV is the elucidation of the requirement of the AAA-ATPase p97 for ligand-dependent nuclear localization of intact receptor. Biochemical data is supplied to support this finding.

CHAPTER II

MATERIALS AND METHODS

Cell Culture and Transfections

Transient transfection for expression in mammalian cells was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two μg of expression plasmid was used per 60mm dish. Transient expression of full-length EGF receptor in NIH3T3 cells was carried out using a DNA: Fugene6 (Roche) ratio of 1.5μg:4.5μl, according to the manufacturer's instructions. To assess ligand-dependent EGFR activation, transfected cells were serum starved overnight and treated with 50ng/ml EGF.

Reagents, Antibodies, Cell lines, and Plasmids

NIH3T3, Cos-7, and 293 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals) at 37°C at 5% CO_2. EGF was purchased from R&D Systems.

Monoclonal anti-Flag was purchased from Sigma. Polyclonal anti-EGFR pY1173 was purchased from Santa Cruz Biotechnologies. Polyclonal anti-EGFR was purchased from Millipore. Anti-EGFR Ab-3 was purchased from LabVision. Monoclonal anti-p97 was purchased from Fitzgerald Industries. The respective

HRP-conjugated secondary antibodies were purchased from Zymed. Alexa-488 goat-anti mouse was purchased from Molecular Probes. For immunoblot detection, enhanced chemiluminescence reagent from Perkin Elmer Life Sciences was used. Stripping and reprobing of blots was performed according to the manufacturer's recommendations.

The plasmid encoding EGFR-ICD-flag was cloned as previously described (Thiel and Carpenter 2007) and its sequence verified. Site-directed mutagenesis was employed to make all mutants with the use of PfuUltra polymerase (Stratagene). Flag-EGFR plasmid was a gift from Tony Burgess (Ludwig Institute for Cancer Research) and was fully sequenced. The pBABE retroviral vectors used in EGF receptor expression for soft agar and xenograft assays were purchased from Addgene. Site-directed mutagenesis of pBABE-EGFR was employed to make pBABE-EGFR-V665M. All shRNA constructs were purchased from Open Biosystems. All mutations were confirmed by fully sequencing each construct.

Cell Lysis and Immunoblotting

Cells at 80-90% confluence were serum-starved and either untreated or treated with EGF as indicated, and then washed with phosphate-buffered saline (PBS). Cells were scraped and lysed in ice-cold lysis buffer containing 1% Triton, 10% glycerol, 50mM Hepes, pH 7.2, and 100mM NaCl, 1mM EDTA, 1mM sodium orthovanadate, 1mM phenylmethylsulfonylfluoride, 10ug/mL aprotinin, and 25ug/mL leupeptin. Lysates were cleared by centrifugation at 13,000 x g for

10 minutes at 4°C. SDS sample buffer was added to lysates and samples were boiled for 5 minutes. Samples were then subjected to SDS-PAGE on 8% polyacrylamide gels, transferred to polyvinylidene fluoride membranes and immunoblotted.

Nuclear and non-nuclear fractions were prepared from 293 cells. Cells were washed with phosphate buffered saline. One mL of Buffer B (Buffer A (10mM Hepes, pH 7.9, 1.5mM $MgCl_2$, 10mMKCl, 1mM EDTA, 1mM sodium orthovanadate, 1mM phenylmethylsulfonylfluoride, 10ug/mL aprotinin, and 25ug/mL leupeptin) plus 1% NP-40) was added per 10cm dish and cells were scraped into microfuge tubes. Tubes were incubated on ice and vortexed at ~50% speed 3 times for a duration of 5 seconds per vortex within a 10 minute period. Cells were subjected to centrifugation at 400 x g for 4 minutes at 4ºC. 800 µL of supernatant was transferred to a fresh microfuge tube and rocked for 30 minutes 4ºC. The non-nuclear fraction was collected after centrifugation for 10 minutes at 13,000 x g. The pellet resulting from the initial centrifugation step was washed with Buffer B twice, then pellets were incubated 30 minutes on ice in of 50uL Buffer C (65% Buffer A, 25% glycerol, 10% 5M NaCl). The soluble nuclear fraction was collected from the resulting supernatant after centrifugation for 5 minutes at 13,000 x g.

Retrovirus-Mediated Gene Transduction and Colony Formation Assay

The pBABE-puro retroviral vector containing the coding sequences for wild-type EGFR, or receptor mutants V665M, L834R, or D813A were used to produce virus-containing supernatants from transfected Phoenix cells. Viral supernatant was used to transduce NIH3T3 cells in the presence of 5μg/ml polybrene. Equal amounts of retrovirus containing wild-type EGFR, or receptor mutants V665M, L834R, D813A, or vector alone were used for infection. NIH 3T3 cells stably expressing wild-type EGFR or receptor mutants were selected using 2ug/mL puromycin for 7 days. Cells expressing equal concentrations of receptor were sorted via FACS by staining with a non-ligand interfering ectodomain antibody (anti-EGFR Ab-3, LabVision) and then seeded at 8×10^3 cells per well in the 0.4% top layer of a 12-well plate. Cells were cultured in 10% FBS with or without 50ng/ml EGF. Medium was changed every two days and colonies were counted at 3 weeks using an Oxford Optronix Gelcount.

In Vivo Tumorigenicity

To determine the capacity of NIH3T3 cells to form tumors *in vivo* when stably expressing pBabe-puro vector, wild-type, or V665M EGFR, nude mice (4 mice per cell type) received four dorsal subcutaneous injections, one in each quadrant, with either 1.25×10^5, 2.5×10^5, 5×10^5, or 1×10^6 tumor cells in 200 µl PBS. Tumor growth was then analyzed over time, as plotted in Figure 5. Three months post tumor cell injection, the mice were sacrificed and the number and

size of tumors were evaluated. Tumor volumes were calculated using the following formula: tumor volume (mm^3) = (length x $width^2$)/2 (Chen, Su et al. 2005). All animal protocols were approved by Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

CHAPTER III

THE JUXTAMEMBRANE REGION OF THE EGF RECEPTOR FUNCTIONS AS AN ACTIVATION DOMAIN

INTRODUCTION

The 38 residue juxtamembrane (JM) region of the EGF receptor contains a variety of signaling and sorting motifs. There are two sites of threonine phosphorylation within the JM region (Figure1). T654 is defined as a protein kinase C (PKC) phosphorylation site and T669 a mitogen-activated protein (MAP) kinase site (Davis 1988; Takishima, Griswold-Prenner et al. 1991). T654 and T669 are phosphorylated with inhibitory consequences for kinase activity (Heisermann, Wiley et al. 1990; Welsh, Gill et al. 1991). Heisermann et al (1990) showed that mutation of T669 in full-length receptor resulted in decreased endocytosis and tyrosine phosphorylation of cellular substrates. Moreover, Welsh et al. (1991) demonstrated that not only is phosphorylation of T654 required for TPA-mediated attenuation of EGF-induced tyrosine phosphorylation of cellular substrates, but T654 is also required for attenuation of morphological changes in NR6 cells ectopically expressing wild-type or T654A EGF receptors. In this case, TPA-induced lamellopodia formation was more extensive and rapid in cells containing the T654A mutant receptor.

Four motifs for receptor sorting have been defined in the JM region (Figure 1). Two basolateral sorting motifs are present and encoded by either a

dileucine motif (L658-L659) or a PXXP motif (P667-P670) (He, Hobert et al. 2002). A lysosomal sorting signal has been defined (L679-L680) (Kil, Hobert et al. 1999; Song Jae Kil 2000), as well as a tripartite nuclear localization signal (R645-R647, R651-R653, R656-R657) (Hsu and Hung 2007).

In addition to sorting motifs, a polybasic region (R645-Q660) within the JM has been proposed to modulate the activity of the kinase by acting as an 'electrostatic engine' (McLaughlin, Smith et al. 2005). Under basal conditions, this model predicts that the polybasic region of the JM electrostatically interacts with the plasma membrane (PM). Upon ligand-induced receptor dimerization, transient increase in intracellular calcium leads to both reduction of electrostatic attractions as well as an increase in calmodulin levels. Peptide studies have demonstrated that the JM interacts with lipid vesicles and that calmodulin, which associates with the EGF receptor JM, competitively inhibits JM peptide interaction with lipid vesicles (McLaughlin, Smith et al. 2005). The electrostatic engine model predicts that the interaction of the JM polybasic region with the PM is autoinhibitory and that the calcium/calmodulin-induced dissociation of the JM from the PM leads to autophosphorylation of the receptor.

In previous studies using large deletion mutations, a portion of the JM region was required for kinase activation (Thiel and Carpenter 2007). Thus, the JM region of EGFR plays an activating role in the control of kinase activity and this contrasts with the autoinhibitory function described for JM regions of other RTKs, such as EphB2 and Flt3 (Hubbard 2004). The conventional mechanism of JM-mediated tyrosine kinase autoinhibition is postulated to be due to the steric

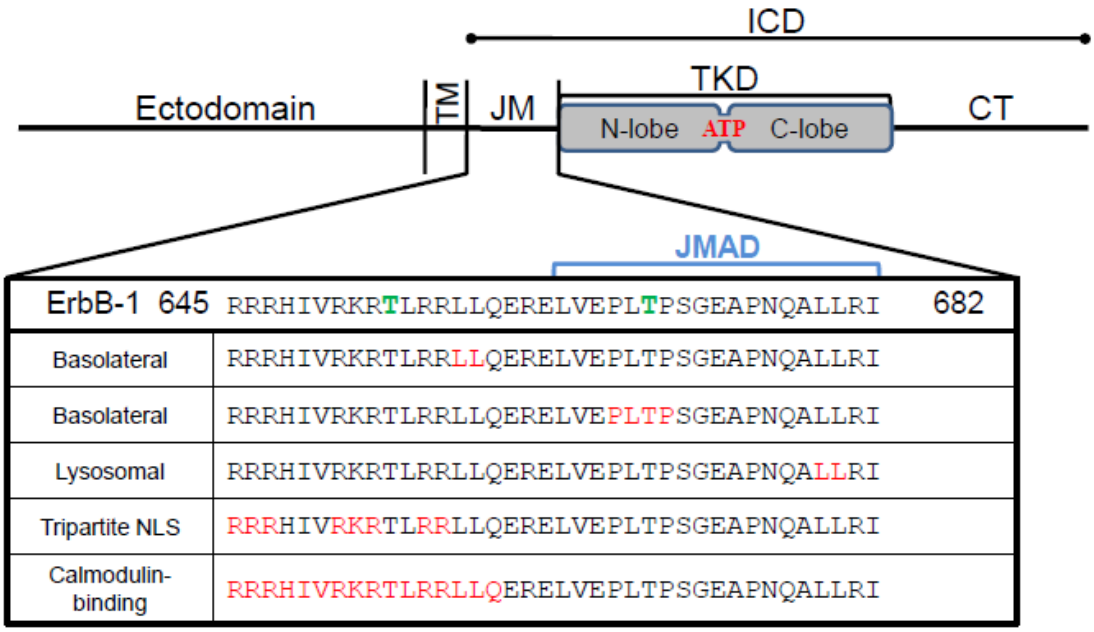
hindrance of the nucleotide binding site by the JM region. This mode of autoinhibition is relieved upon phosphorylation of tyrosine residues within the JM, resulting in the movement of the JM away from the ATP-binding site.

In this chapter, I define the minimal JM activation domain (JMAD) using alanine scanning mutagenesis, and evaluate reported lung cancer mutations in this region for their influence on EGFR activity. I also discuss a crystal structure of the EGFR TKD that contains the entire JM region. This structure, produced by the Lemmon laboratory, illustrates the key interactions responsible for JMAD function in TKD activation, and suggests structural mechanisms for activation of EGFR by JM mutations in non-small cell lung cancer (NSCLC).

RESULTS

Scanning Alanine Mutagenesis of the EGF Receptor Juxtamembrane Region.

Previously published data showed that deletions in the JM region abolish both autophosphorylation of an intracellular domain (ICD) construct and EGF-induced activation of intact EGFR overexpressed in Cos-7 and NIH3T3 cells, respectively (Thiel and Carpenter 2007). Deleting residues 645-662 or 645-676 of the JM region reduced ICD autophosphorylation in cells by ~95%. Thiel and Carpenter proposed that these deletions indicated the presence of an activation domain within the JM region.



ErbB-1	645 RRRHIVRKRTLRRLLQERELVEPLTPSGEAPNQALLRI	682
Basolateral	RRRHIVRKRTLRRLLQERELVEPLTPSGEAPNQALLRI	
Basolateral	RRRHIVRKRTLRRLLQERELVEPLTPSGEAPNQALLRI	
Lysosomal	RRRHIVRKRTLRRLLQERELVEPLTPSGEAPNQALLRI	
Tripartite NLS	RRRHIVRKRTLRRLLQERELVEPLTPSGEAPNQALLRI	
Calmodulin-binding	RRRHIVRKRTLRRLLQERELVEPLTPSGEAPNQALLRI	

Figure 1. Schematic of EGF Receptor JM Sorting Sequences. Domains and regions of the EGF receptor are indicated. The residues comprising the JM are numbered 645-682 and the PKC and MAPK phosphorylation site T654 and T669 are shown in green. Receptor sorting sequences are aligned with the JM sequence. A polybasic region extends from R645-R667.

JM residues 645-671 are absent from crystal structures of the EGFR TKD, which utilize constructs beginning at residue 672 (Stamos, Sliwkowski et al. 2002; Wood, Truesdale et al. 2004; Zhang, Gureasko et al. 2006). To determine which residues within the JM region contribute to tyrosine kinase activation, I employed scanning alanine mutagenesis of the 38 residue region in the context of a Flag epitope-tagged ICD construct (Figure 2A). Since Thiel and Carpenter previously showed that mutating K721 (thus abrogating ATP-binding) abolished *in vivo* tyrosine autophosphorylation of EGFR ICD constructs, this argues that measurement of phosphotyrosine incorporation into the ICD reflects intrinsic kinase activity levels. Therefore, the influence of mutations on ICD autophosphorylation only reflects the effects on TKD activation. Indirect effects that arise from mutating JM trafficking motifs will not complicate the interpretation of these ICD studies as they might in studies of the intact receptor (Kil, Hobert et al. 1999; Song Jae Kil 2000; Lin, Makino et al. 2001; He, Hobert et al. 2002).

Of the 36 alanine substitutions made in the JM region, 19 led to a significant reduction (≥50%) in ICD tyrosine phosphorylation (Figure 2B). Fifteen of these 'sensitive' positions are located in the C-terminal portion of the JM (Figure 2B, Figure 3), between residues 664 and 682. This is within a region of the JM encoded by exon 18 (which also encodes a portion of the N-lobe of the kinase domain). Within this sequence, T669 (a known MAP kinase phosphorylation site: see above) is the only site at which alanine substitution does not impair kinase activity. Interestingly, four of the loss-of-function mutations are either similar (L664A, V665A) or identical (P675A, L680A) to

changes present in the JM region of ErbB-3 (Figure 2A), a member of the EGFR family having an inactive tyrosine kinase domain (Guy, Platko et al. 1994). The boundary between exon 17 and exon 18 is conserved in all 4 ErbB receptors, and the four receptors share more identity in the part of the JM region encoded by exon 18 than by exon 17 (Figure 2A).

Analysis of JM Mutations in Full-Length EGFR.

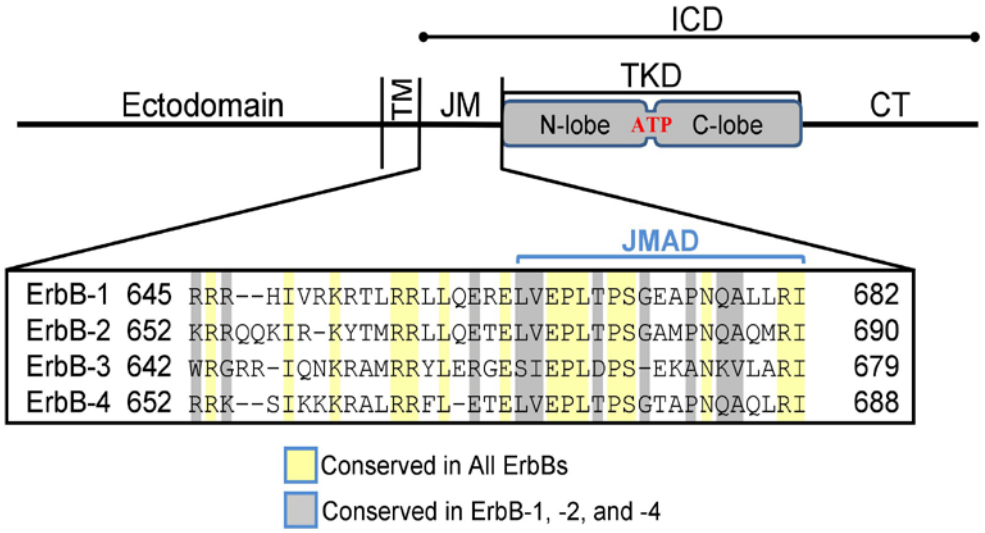
Three conservative JM mutations that span the entire JM region were analyzed in full-length EGFR: V650A, V665A, and L680A (Figure 2C). Also, a catalytically inactive K721R EGFR was included to establish a baseline for loss-of-function. V650A was chosen as a control for replication of the ICD data in full-length receptor because the level of tyrosine phosphorylation of the mutant ICD was equivalent to that of wild-type ICD (Figure 2B, Figure 3). Each EGFR mutant was transiently expressed in NIH3T3 cells, and receptor tyrosine phosphorylation in the absence and presence of EGF at Y1173, a known major autophosphorylation site (Downward, Waterfield et al. 1985), was measured by western blotting. The V650A mutant resembles wild-type EGFR in EGF-dependent autophosphorylation, consistent with the failure of this mutation to reduce ICD autophosphorylation (Figure 2C and 2B). By contrast, autophosphorylation of the V665A or L680A mutants is significantly compromised, as in the ICD context (Figure 2B), and autophosphorylation of K721R EGFR is undetectable. To control for the possibility that the results obtained with V665A and L680A EGFR mutants arise from impaired receptor trafficking to the cell surface, Alexafluor-

conjugated EGF was incubated with 293 cells expressing each mutant or wild-type EGFR and cell-surface fluorescence was assessed, using flow cytometry. All full-length receptor mutants were expressed on the cell surface at levels within 10% of the wild-type receptor (Figure 4A).

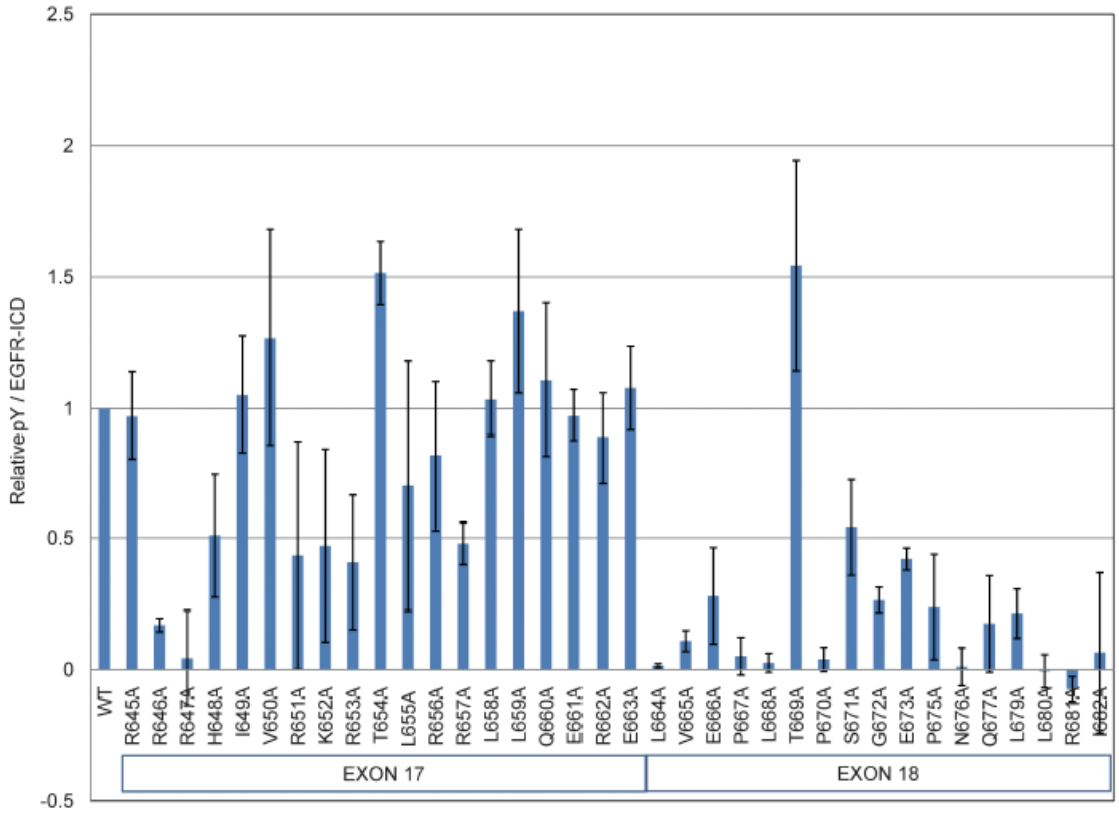
Effects of Threonine Phosphorylation within the JM Region.

The JM region contains two threonine residues that are phosphorylated in presumed regulatory feedback loops. A previous study showed (Thiel and Carpenter 2007) that a phosphomimetic T654D mutation reduces ICD autophosphorylation by 50% compared to wild-type, whereas a T654A mutation elevates ICD phosphorylation (Figure 1B). As shown in Figure 5A, a phosphomimetic T669D mutation also prevented ICD tyrosine phosphorylation, whereas a T669A mutation lead to elevated tyrosine phosphorylation compared to the wild-type ICD (Figures 5A and 1B). Thus, phosphorylation of T669 is likely to exert the same influence on EGFR ICD activity as T654 phosphorylation, supporting a feedback mechanism for EGFR regulation by post-translational modification of T669 within the putative JM activation domain.

A



B



Red Brewer et al. Mol Cell 34: 641-651 (2009)

C

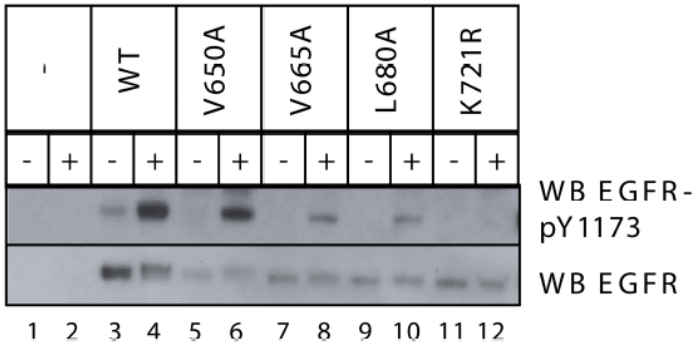


Figure 2. Effect of Scanning Alanine Mutagenesis of the JM Region on Tyrosine Phosphorylation. A. Schematic of full-length EGFR, with the transmembrane (TM), tyrosine kinase domain (TKD), and carboxy-terminal (CT) regions marked**.** The intracellular domain (ICD) construct includes the JM, TKD, and CT regions. An alignment of JM region sequences across the human ErbB receptor family is shown, with the putative JM activation domain (JMAD) indicated. **B.** Scanning alanine mutagenesis of the JM region using ICD-flag constructs. Cos-7 cells transiently expressing wild-type or mutated ICD-flag constructs were analyzed by Western blotting and densitometry for both phosphotyrosine content and flag-epitope expression. The graph demonstrates the ratio of phosphotyrosine to flag expression for each construct relative to WT. Results represent the means of 2 experiments ± standard deviation. **C.** Effect of JM alanine substitutions on tyrosine autophosphorylation of full-length EGFR. NIH3T3 cells transiently expressing full-length EGFR with the indicated mutations were serum-starved and then treated (+) or not (-) with EGF for 10 min. Cell lysates were subjected to SDS-PAGE and immunoblotting, detecting phospho-EGFR with anti-EGFR-pY1173 (upper panel) and receptor levels with anti-EGFR (lower panel).

Red Brewer et al. Mol Cell 34: 641-651 (2009)

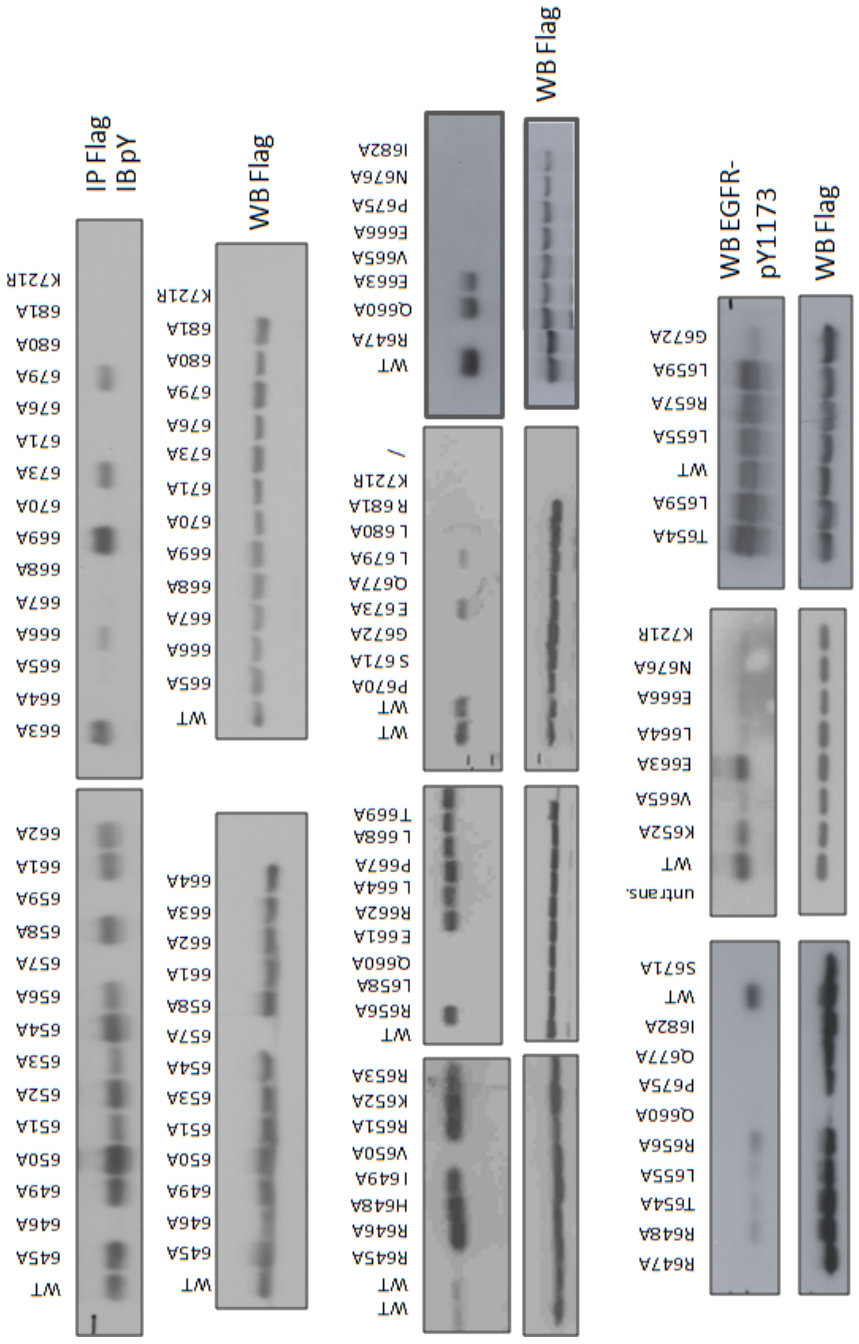


Figure 3. Scanning Alanine Mutagenesis of the JM Region Using ICD-flag Constructs. Cos-7 cells transiently expressing wild-type or mutant ICD-flag constructs were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine and anti-Flag (as described in Experimental Procedures). The blots were then analyzed densitometrically for both phosphotyrosine and flag-epitope expression.

Red Brewer et al. Mol Cell 34: 641-651 (2009)

A

EGFR	Alexa-EGF bound (normalized to WT)
WT	1.00
V665A	0.98
L680A	0.90
V665M	0.97

B

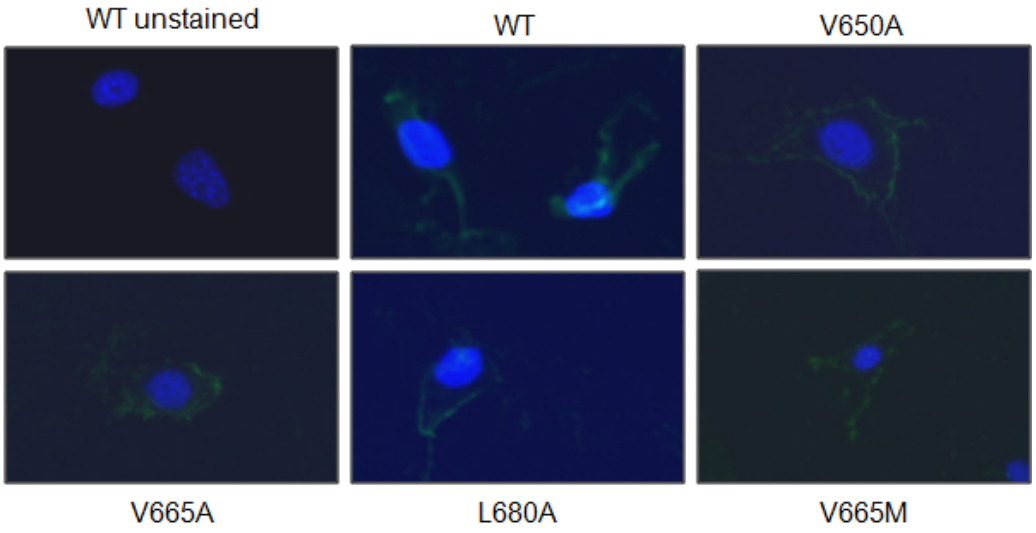


Figure 4. Cell Surface Expression of Full-length EGF Receptor Mutants. A. 293 cells transiently expressing equivalent levels of N-terminal Flag-tagged WT, V665A, L680A, or V665M EGF receptors were treated with Alexa-EGF and analyzed using flow cytometry. Geometric mean fluorescence values were collected and Alexa-EGF binding was quantified. All mutants were normalized to WT. **B.** NIH3T3 cells transiently expressing equivalent levels of the constructs indicated above were stained with anti-EGFR followed by Alexa488-labeled anti-mouse. Cells were fixed with 4%PFA and mounted with glass coverslips. Images were acquired using a Zeiss fluorescence microscope (see Supplemental Experimental Procedures).

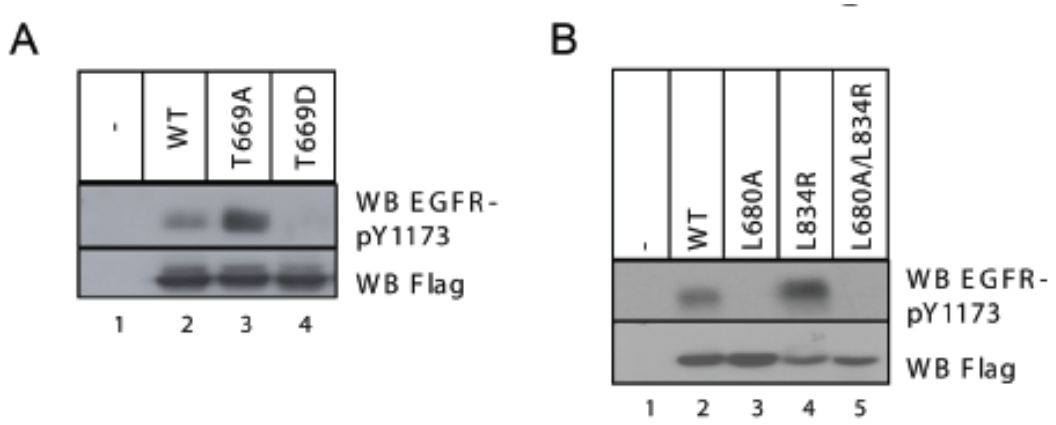


Figure 5. Effects of Mutations at JM Phosphorylation Sites. A. Effect of a phosphomimetic mutation at T669 on ICD tyrosine Phosphorylation. The indicated flag-tagged ICD constructs were transiently expressed in Cos-7 cells. Lysates were subjected to SDS-PAGE and immunoblotting as in Figure 1C, but using anti-Flag to normalize for ICD expression levels. **B.** Regulation of constitutively active EGFR-L834R by a JM loss-of-function mutant. 293 cells transiently expressing indicated ICD constructs were lysed and subjected to SDS-PAGE and immunoblotted with anti-EGFR-pY-1173 and anti-Flag.

Regulation of L834R Tyrosine Phosphorylation by JM Mutation.

Clinical studies of non-small cell lung cancer (NSCLC) have identified a number of mutations in the EGFR TKD that are associated with increased sensitivity to EGFR-targeted tyrosine kinase inhibitors (Sharma, Bell et al. 2007). Many of these mutations cause constitutive (ligand-independent) activation of the EGF receptor when studied in reconstituted cellular systems (Jiang, Greulich et al. 2005; Choi, Mendrola et al. 2007), and appear to do so by disrupting autoinhibitory interactions within the TKD (Zhang, Gureasko et al. 2006). The L834R mutation, located in the kinase activation loop, is one of the most clinically frequent and well characterized examples. To determine whether EGFR activation by such mutations requires JM-mediated interactions, I combined the L834R mutation with a loss-of-function JM region mutation (L680A). A doubly mutated ICD construct containing L834R plus the JM L680A mutation was expressed in Cos-7 cells. Figure 5B shows that kinase activation due to the L834R mutation was blocked by the L680A mutation, demonstrating that the activated kinase remains dependent on JM function.

Activating EGFR JM Mutations in Non-Small Cell Lung Cancer.

In addition to the TKD mutations, several publications have described relatively rare EGFR exon 18 JM point mutations in NSCLC patients (Chou, Chiu et al. 2005; Tsao, Sakurada et al. 2005; Pallis, Voutsina et al. 2007). However, the impact of these mutations on receptor activity was not assessed, and in some

cases normal patient tissue was not examined for the presence of the mutation. Thus, the relationship of these mutations to NSCLC is currently not clear.

To determine whether these patient-derived JM mutations effect receptor kinase activity, equivalent mutations were made in ICD constructs and expressed in Cos-7 cells (Figure 6A). The L834R mutant was also analyzed as a positive control. Most of the patient-derived JM mutations (L664P, P667S, L668P, P670L/S, and N676D) reduced ICD autophosphorylation, either suggesting that they may not be relevant in NSCLC, or that mutation results in misfolding of the ICD (Figure 6A). However, two mutations (V665M and L679F) significantly increased ICD tyrosine phosphorylation, to levels similar to those produced by the L834R mutation.

I next introduced one gain-of-function mutant, V665M, which was not included in previously published crystal structures, as well as two loss-of-function mutants, L668P and P670L, into full-length EGFR, and measured tyrosine phosphorylation of transiently expressed receptor in NIH 3T3 cells treated or not with EGF (Figure 6B and 6C). Consistent with the ICD studies, the V665M mutation promotes ligand-independent EGFR autophosphorylation such that V665M EGFR is as phosphorylated in the absence of EGF as the activated wild-type receptor in the presence of EGF. This effect cannot be explained by differences in cell-surface expression of the mutant compared to wild-type receptor (Figure 4A). Thus, the V665M mutation resembles other activating NSCLC mutations in its effects on full-length EGFR (Jiang, Greulich et al. 2005; Choi, Mendrola et al. 2007), suggesting that it could represent an oncogenic JM

mutation with relevance in NSCLC. By contrast, the L668P mutation reduced EGF-induced receptor autophosphorylation, consistent with its effect in the ICD context. These biochemical data suggests that for the L668P mutant NSCLC tumor, the observed disease may have been due to an EGF receptor-independent mechanism. In the presence of EGF, the P670L mutant was tyrosine phosphorylated at a level equivalent to that of wild-type receptor (Figure 6C). This result was inconsistent with the observed loss of autophosphorylation in the ICD system, suggesting a possible effect of this proline substitution on ICD stability (Figure 2B and 3).

V665M EGFR Promotes Cellular Transformation and Tumorigenesis.

The hallmarks of cellular transformation in culture are growth in the absence of serum, loss of contact inhibition, and anchorage-independent growth (Hanahan and Weinberg 2000; Ruddon 2007). To evaluate the proliferative and oncogenic potential of the V665M mutation, I tested NIH3T3 cells stably expressing wild-type, V665M, L834R, or D813A (catalytically-inactive) EGFR for colony formation in soft agar in the absence and presence of EGF. In the presence of EGF the V665M EGFR mutant supports anchorage-independent growth more effectively than the wild-type receptor (Figure 7A). Moreover, cells expressing V665M EGFR formed more colonies following EGF addition than cells expressing the well-described NSCLC mutant L834R. Neither the V665M nor the L834R mutant-expressing NIH 3T3 cells formed a substantial

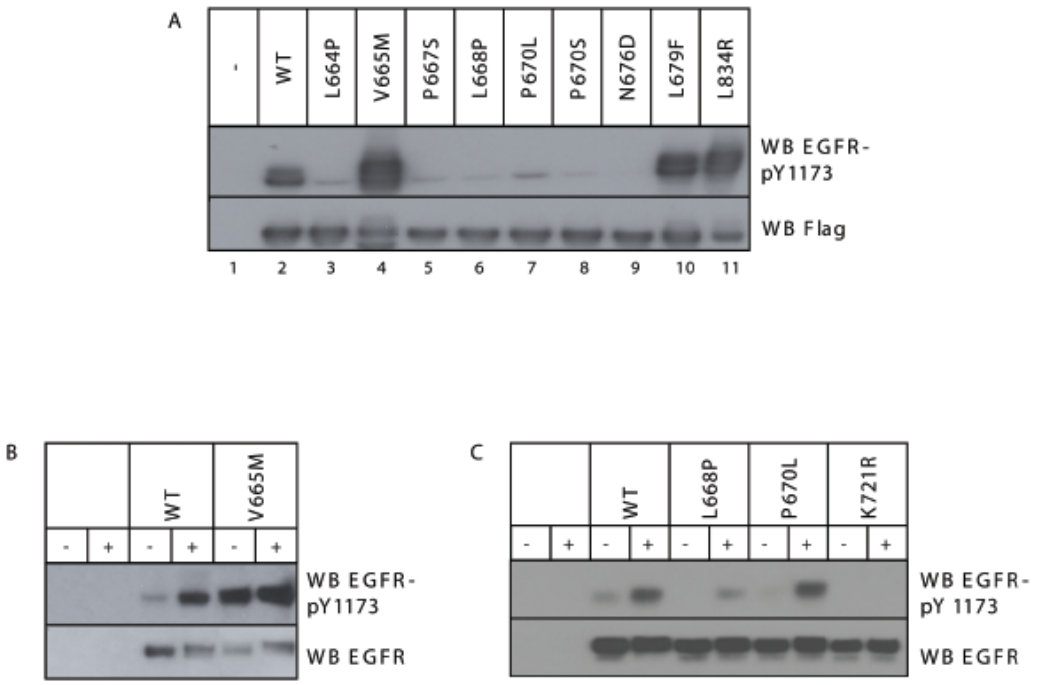


Figure 6. Effects of Clinically-Observed JM Mutations on EGFR Activity. A. The noted clinically-observed JM mutations were introduced into the Flag-tagged EGFR ICD. The resulting constructs were transiently expressed in Cos-7 cells, and ICD autophosphorylation was assessed as described in Figure 1. **B.** and **C.** The V665M, L668P, and P670L mutations were also introduced into full-length EGFR, and the influence on EGF-dependent (+) and EGF-independent (-) EGFR autophosphorylation was assessed as described for Figure 1C. The K721R inactivating mutation was included as a negative control (**C**).

Red Brewer et al. Mol Cell 34: 641-651 (2009)

number of colonies in the absence of EGF, although they formed approximately 2 and 4 times more colonies than untreated NIH 3T3 cells expressing wild-type receptor, respectively (Figure 7A). The NIH 3T3 parental cell type was selected for its low level of endogenous EGF receptor (~5,000 receptors/cell) compared to other cell types that typically express moderate levels of EGF receptor, as well as other ErbB family members. Importantly, cells expressing the vector only form an equivalent number of colonies to wild-type expressing cells in the absence of EGF, consistent with the requirement of EGF receptor tyrosine kinase activity for proliferative response.

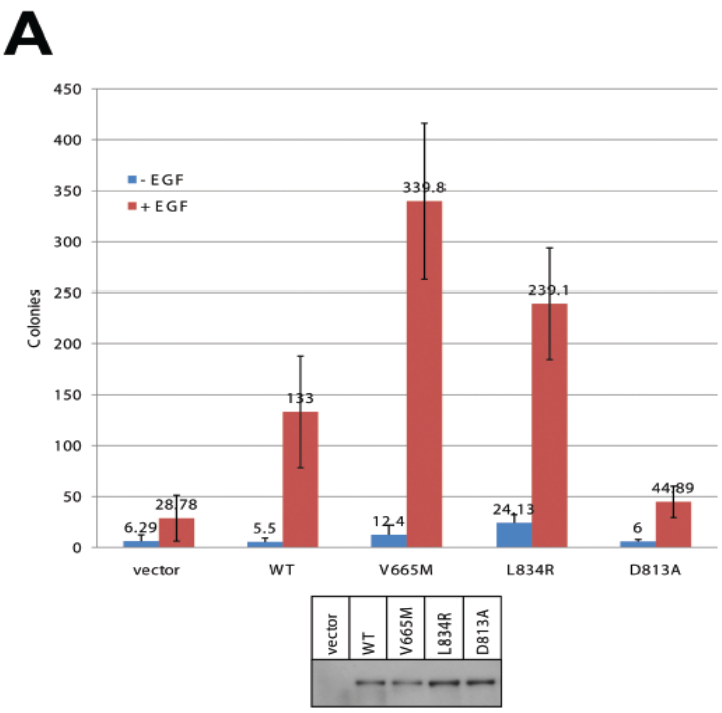
In collaboration with Dr. Ambra Pozzi, I sought to determine whether the observed increase in anchorage-independent growth produced by the V665M EGFR *in vitro* reflects oncogenic transformation *in vivo*. Dr. Pozzi performed subcutaneous injections of NIH3T3 cells stably expressing equivalent levels of wild-type or V665M EGFR into nude mice. Three months after injection, cells expressing V665M EGFR formed more (and larger) tumors than cells expressing wild-type EGFR, regardless of the number of cells injected (Figure7B). These results indicate that the JM activation domain is a significant factor in the regulation of EGF receptor kinase activity at the biological, as well as biochemical, level.

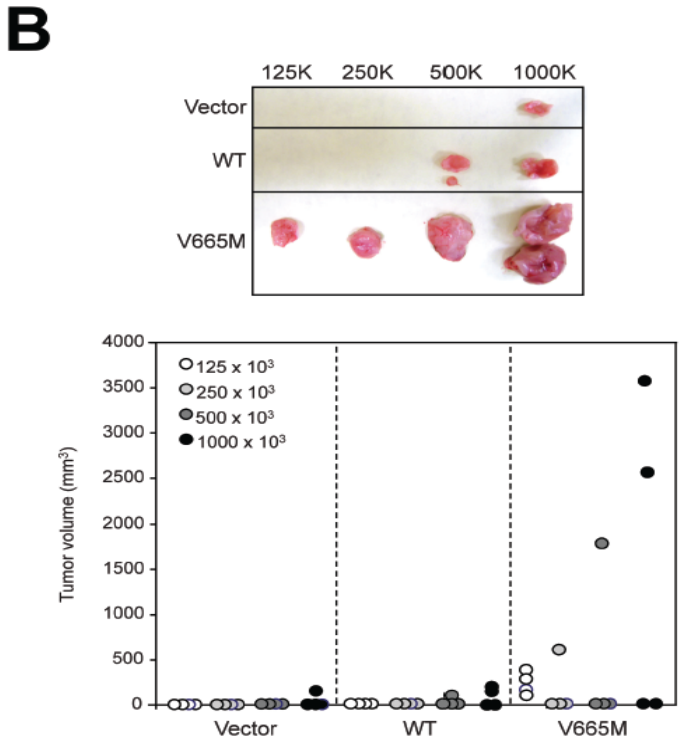
Due to the increase in oncogenic potential mediated by the V665M mutation (Figure 7), attempts to understand potential signaling differences between the wild-type and V665M mutant EGF receptor were made by assessing both EGFR tyrosine autophosphorylation and Stat-3 activation. Recent data

suggests that EGF receptor mutations within the TKD of EGF receptor can mediate constitutive Stat-3 activation via an IL-6 autocrine pathway in different NSCLC lines, the end result of which is increased cell survival (Haura, Zheng et al. 2005; Gao, Mark et al. 2007). 293 cells transiently expressing EGF receptor containing the V665M mutation resulted in an increase in basal and ligand-induced Stat-3 activation compared to wild-type receptor (Figure 8). No significant differences were observed in phosphorylation of Tyr 992, 1045, or 1068. These data are consistent with a V665M mutant-dependent pStat-3, which in other systems is reported to result in a prosurvival phenotype.

Mechanism of EGFR Activation by the V665M Mutation.

Zhang *et al*. (2006) described a mechanism for activation of the EGFR TKD in which the C-lobe of a 'donor' monomer contacts the N-lobe of an 'acceptor' monomer in the asymmetric dimer (Figure 11B). The resulting conformational changes in the acceptor monomer lead to its allosteric activation. I took advantage of mutations (Zhang, Gureasko et al. 2006) that force receptor molecules to act solely as donor monomers (I682Q) or acceptor monomers (V924R) to determine whether the V665M-mutated EGFR ICD functions more effectively than wild-type as donor, acceptor, or both.





Red Brewer et al. Mol Cell 34: 641-651 (2009)

Figure 7. EGFR-V665M is Transforming in NIH3T3 Cells. A. NIH3T3 cells stably expressing the indicated wild-type or mutated forms of EGFR (or pBabe-puro vector only) were seeded at a density of $8x10^3$ cells per well in a standard colony forming assay (see Experimental Procedures). Cells were incubated for 3 weeks in the absence or presence of 50ng/ml EGF, and colonies were then counted. The results are presented as the means of 3 experiments ± standard deviation. To control for possible differences in expression level of the mutants, NIH3T3 stably expressing each mutant were lysed and analyzed by SDS-PAGE and immunoblotting with anti-EGFR. **B.** Upper panel: Example of tumors isolated from nude mice 3 months after s.c. injection with NIH3T3 cells stably expressing wild-type or V665M EGFR or the pBabe-puro vector. Lower panel: Volumes of tumors derived from the cells indicated above were evaluated 3 months after s.c. injection. Open circles represent single mice with a total of 4 mice per cell type analyzed. Cells for xenograft assay created by Monica Red Brewer. Subcutaneous injection, tumor collection, and measurement by Ambra Pozzi.

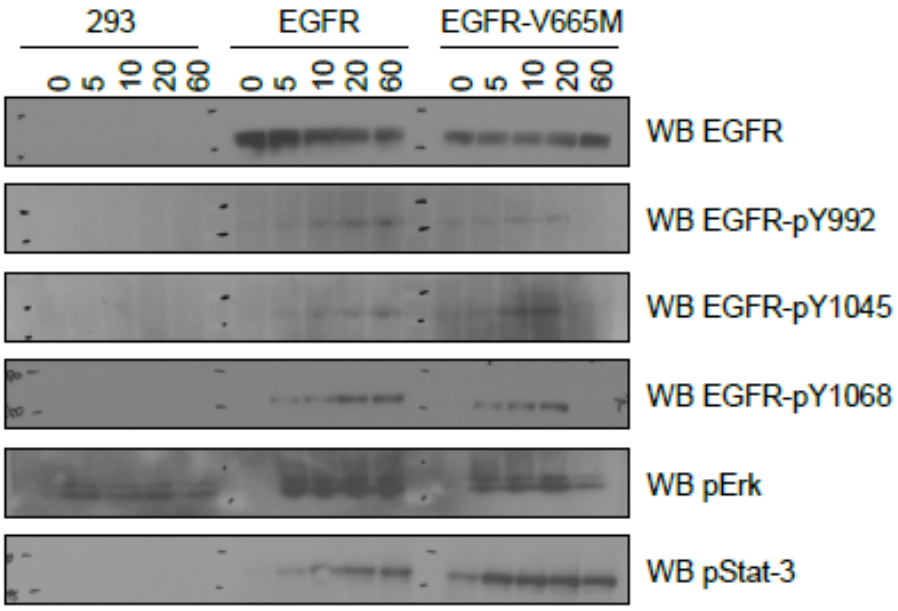


Figure 8. The EGF Receptor V665M Mutation Mediates Constitutive Activation of Stat-3. 293 cells transiently expressing either wild-type or mutant EGF receptor were serum-starved an treated or not for the indicated times (in minutes). Cells were then lysed and lysates resolved by SDS-PAGE. Membranes were western blotted with the indicated antibodies.

Red Brewer et al. Unpublished data (2009)

The donor ICD was myc-tagged, and the acceptor ICD was FLAG-tagged, and when expressed alone, neither ICD is autophosphorylated (Figure 9, lanes 3 and 4); however, substantial activity was detected when the two were co-expressed (lane 5). Lanes 8-10 (Figure 9) demonstrate that increased ICD autophosphorylation compared to the wild-type level is observed when the V665M mutation is present in the acceptor monomer (V924R/V665M), regardless of whether it is co-expressed with the I682Q donor or the doubly-mutated (I682Q/V665M) donor. When the V924R/V665M acceptor was expressed without a donor monomer (Figure 9: lane 7), there was no activation, indicating that the large increase in activation observed in lanes 9 and 10 requires donor: acceptor interaction. In contrast, coexpression of an I682Q/V665M donor with a V924R acceptor (lane 8) did not increase tyrosine phosphorylation compared to the control (lane 5).

These data establish that the V665M JM mutation enhances the capacity of the ICD to act as an acceptor monomer, but does not greatly influence its ability to function as a donor. This is consistent with the previous finding that an intact JM region is required for the EGFR ICD to function as an acceptor in the asymmetric dimer (Thiel and Carpenter 2007). The V665M mutation seen in NSCLC enhances JM function in this context.

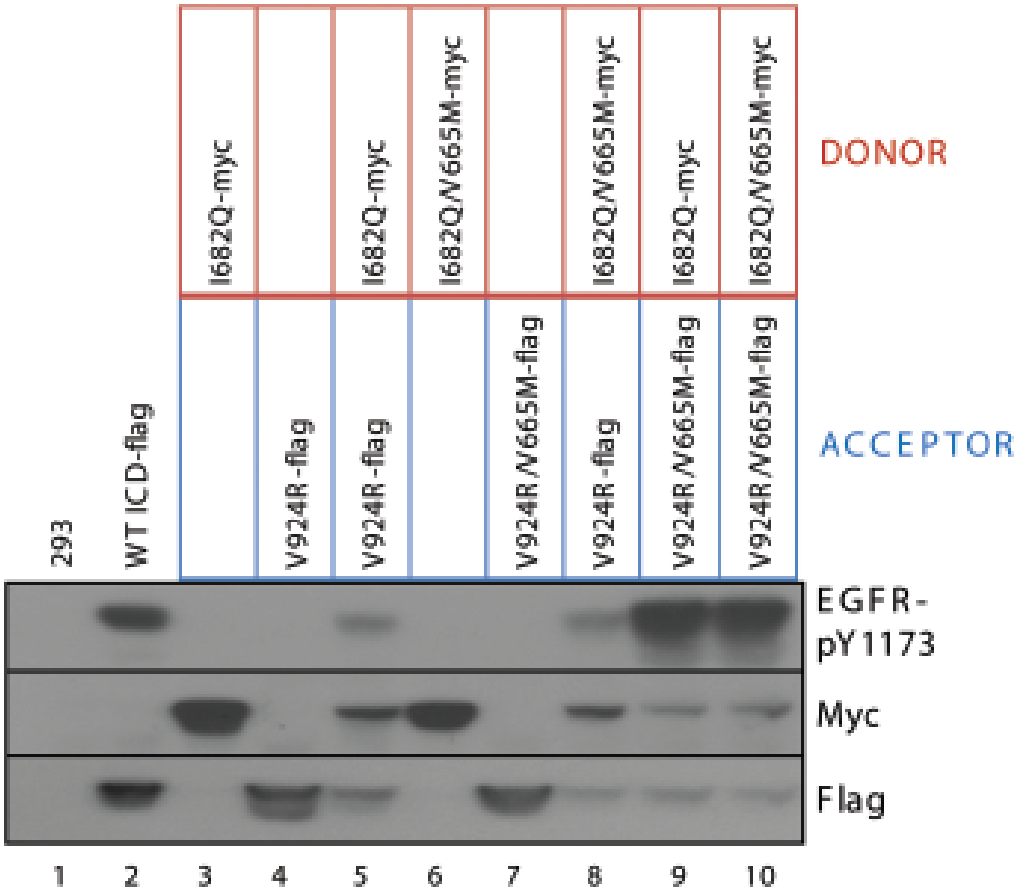


Figure 9. The V665M Mutation Must be Present in the Acceptor Molecule to Activate EGFR. ICD constructs containing mutations that limit them to functioning only as a donor (I682Q) or acceptor (V924R) in the asymmetric TKD dimer were used to assess the mechanism by which the V665M mutation activates EGFR. The noted combinations of ICD constructs were coexpressed in 293 cells as indicated, and lysates were subjected to SDS-PAGE and immunoblotting for phospho-EGFR (with anti-EGFR-pY1173) and for protein levels with anti-Myc, and anti-Flag as appropriate. Autophosphorylation levels are greater than wild-type (lane 2) only when the V665M mutation is present in the acceptor TKD (lanes 9 and 10).

Red Brewer et al. Mol Cell 34: 641-651 (2009)

Sensitivity of the EGF receptor mutant V665M to Small Molecule Inhibition

Due to the novel nature of receptor activation by the juxtamembrane activation domain (JMAD), sensitivity of the activating mutant V665M to the clinically available small molecule inhibitor erlotinib was assessed. Erlotinib, also known as Tarceva, is a reversible TKI that binds to the open conformation of the tyrosine kinase domain within the ATP-binding pocket. 293 cells transiently expressing either wild-type EGF receptor, the activating JM mutant V665M, and the erlotinib-sensitive L834R mutant, were treated with increasing concentrations of erlotinib in the absence or presence of EGF and assessed for inhibition of receptor activation by phosphotyrosine western blot (Figure 10). These data show that the V665M mutant does not demonstrate increased sensitivity to erlotinib compared to wild-type EGF receptor. Complete loss of tyrosine phosphorylation of the wild-type receptor and V665M mutant is observed at a dose of 1uM erlotinib.

DISCUSSION

Crystallographic studies together with mutagenesis and biochemical data indicate that for several RTKs (c-Kit, EphB2, and Flt3) the intracellular JM region plays an autoinhibitory role, sterically hindering substrate access to the nucleotide binding pocket (Wybenga-Groot, Baskin et al. 2001; Chan, Ilangumaran et al. 2003; Griffith, Black et al. 2004). In these RTKs, phosphorylation of key tyrosines within the JM region results in a conformational

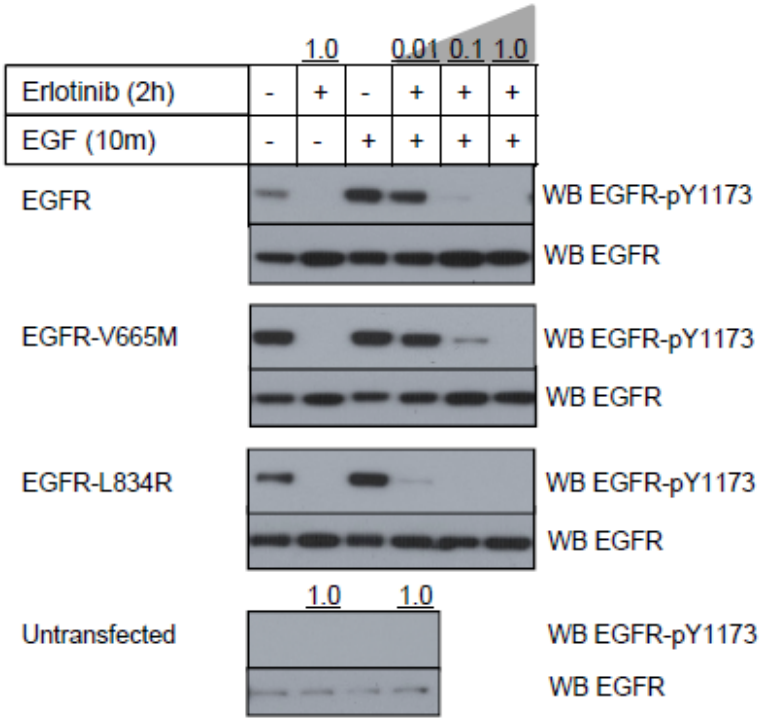


Figure 10. The Non-Small Cell Lung Cancer Mutation EGFR-V665M Shows no Increase in Sensitivity to Erlotinib. NIH3T3 cells transiently expressing either WT EGFR, EGFR-V665M, or the activating EGFR-L834R were serum starved and either treated or not with the indicated concentrations of erlotinib (0.01, 0.1, or 1.0 uM) for 2 hours followed by 50 ng/mL EGF for 10 minutes as indicated. Lysates were subjected to SDS-PAGE and western blotted with anti-EGFR-pY1173 followed by EGFR antibody.

Red Brewer et al. Unpublished data (2009)

shift that reverses the autoinhibition - leading to kinase activation (Binns, Taylor et al. 2000; Mol, Lim et al. 2003).

In contrast, I show here that the JM region (residues 664-682) of the EGF receptor exhibits an activating rather than autoinhibitory role. Unlike the direct occlusion of the kinase active site seen in JM-mediated autoinhibition of other RTKs (Hubbard 2004), the JM-mediated activation of the EGF receptor occurs in an allosteric fashion. Alanine substitutions at most positions in the C-terminal half of the JM region (encoded by exon 18) lead to a loss of kinase activity. I have termed this part of the JM region the JM activation domain (JMAD).

Alanine substitutions at the N-terminal portion of the JM encoded by exon 17 result in a greater degree of variability in terms of tyrosine phosphorylation when compared to wild-type ICD. In general, the N-terminal substitutions do not result in loss of ICD tyrosine phosphorylation. Only two alanine substitutions in this portion of the JM, R646A and R647A, resulted in a severe loss (<25%) in tyrosine phosphorylation (Figure 2B and 3). While the loss-of-function observed for these two alanine mutants could result from misfolding, Jura et al. (2009) have proposed a model that may explain the effect of R646A and R647A on tyrosine phosphorylation. Using structural modeling of the EGF receptor transmembrane domain with existing structural information regarding the TM helical dimers of ErbB-2, the authors propose that R647 lies at the interface of the plasma membrane (PM) and cytosol and electrostatically interacts with the negatively charged head groups of the PM. The positioning of the TM helices and N-terminal residues of the JM are hypothesized to be of paramount

importance in the spatial orientation of the asymmetric TKD dimer, in which an approximate distance of 18-20 Å is required for proper asymmetric alignment and activation of the TKDs.

This model provides an explanation for the loss-of-function observed with the R647A mutant. In this case, disrupting the electrostatic interaction of this basic residue with the phosphates of the PM could certainly have an effect on positioning of the ICDs. However, alignment of the ErbB-2 TM sequence with EGFR R647 shows that this arginine residue is not conserved in ErbB-2, but is a glutamine residue, thus suggesting that the overlay of ErbB-2 and EGFR TM domain provided by modeling may not provide precise information about their spatial distributions, and that there may be slight differences in the distances between the C-terminal TM residues achieved by each of these proteins. My mutational data also implies a role for R646 in this model of spatially controlled TKD dimerization. Whether R646 plays an equivalent role with R647 by electrostatic interaction with the PM is unclear, but would seem quite plausible.

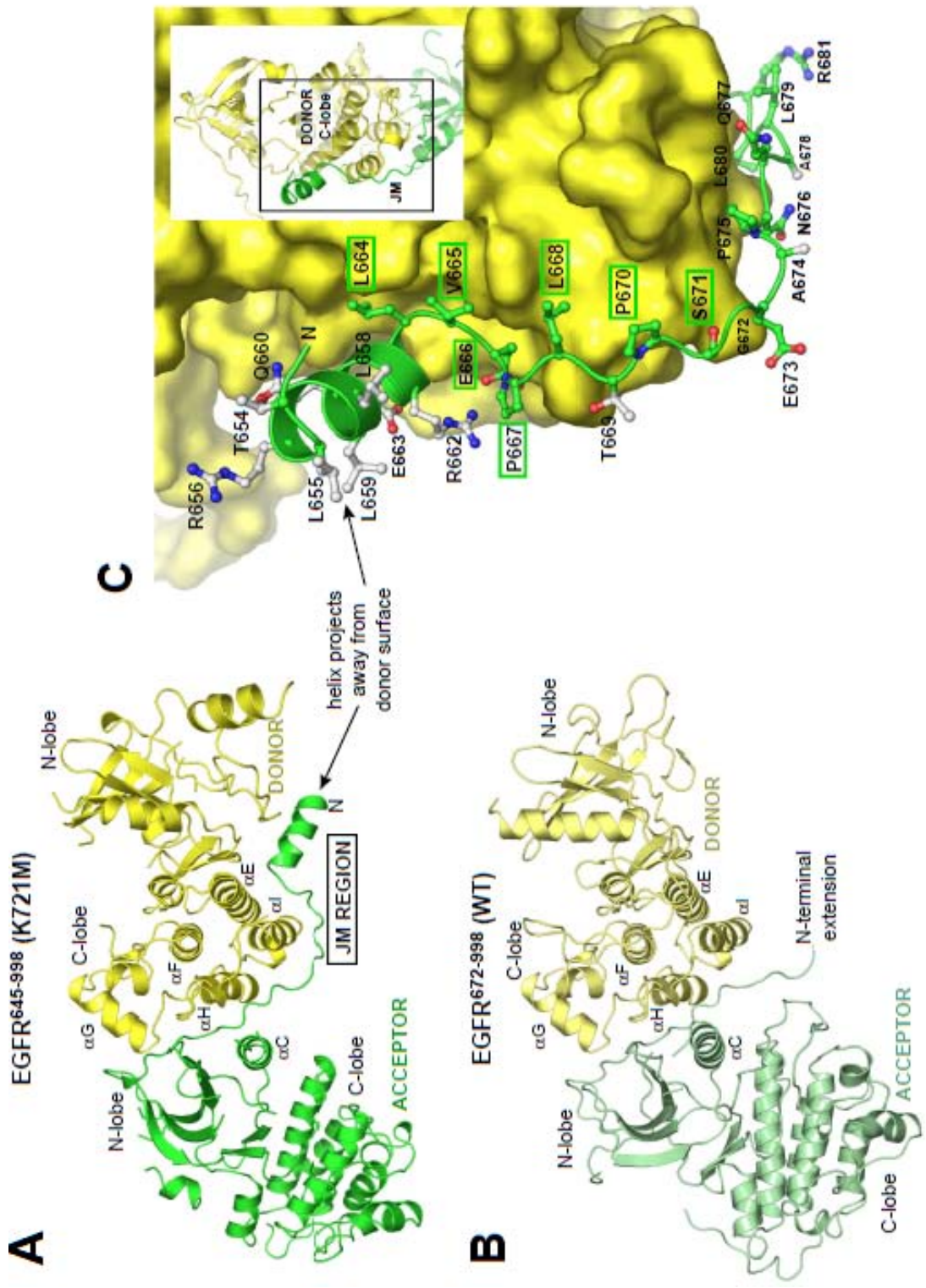
In an effort to understand how the JM region exerts its positive influence on EGFR activation, I collaborated with the Lemmon laboratory to crystallize a form of the EGFR TKD that extends from residue 645 to 998 ($EGFR^{645-998}$), and includes the entire JM region (Red Brewer, Choi et al. 2009). Previous crystallographic studies of EGFR TKD have utilized protein that lacks residues 645-671 (Stamos, Sliwkowski et al. 2002; Wood, Truesdale et al. 2004; Zhang, Gureasko et al. 2006; Yun, Boggon et al. 2007). The best crystals obtained by the Lemmon group diffracted to 2.8Å resolution and were acquired using a form

of $EGFR^{645-998}$ with an inactivating K721M mutation in the kinase active site. Members of the Lemmon Laboratory are responsible for generation of Figures 11 and 12 within this Discussion and have also contributed to the interpretation of the structural information herein. Please refer to Red Brewer et al. (2009) for a more complete account of the structural data presented in this Discussion.

$EGFR^{645-998}$(K721M) formed the same asymmetric dimer as that reported previously (Figures 11A, 12B) for active conformations of the EGFR TKD (Stamos, Sliwkowski et al. 2002; Zhang, Gureasko et al. 2006; Yun, Boggon et al. 2007), and the ErbB4 TKD in both active (Qiu, Tarrant et al. 2008) and inactive (inhibitor-bound) states (Wood, Shewchuk et al. 2008). In this dimer, the C-lobe of the donor monomer (yellow) abuts the N-lobe of the acceptor monomer (green). The binding surface on the acceptor N-lobe includes contributions from the αC helix, the β4/β5 loop, and an N-terminal extension of the N-lobe (marked in Figure 11B) that includes part of the JM region. Rearrangement of these structural elements upon interaction with the donor C-lobe leads to allosteric activation of the acceptor (Zhang, Gureasko et al. 2006). Defined electron density begins at R653, so this structure lacks only the sequence $_{645}$RRRHIVRK$_{652}$ that immediately follows the transmembrane domain. The extended JM region of the acceptor monomer in $EGFR^{645-998}$ appears to 'cradle' the C-lobe of the donor (Figure 11A). The interactions mediated by the JMAD as shown in this structure are consistent with the results of the alanine scanning data in Figure 2B.

Even though most of the donor/acceptor interactions seen in previous active structures (Zhang, Gureasko et al. 2006) are maintained in the EGFR$^{645-998}$(K721M) dimer (Figure 11A), it is apparent that the K721M TKD itself adopts an inactive-like conformation (Wood, Truesdale et al. 2004; Zhang, Gureasko et al. 2006). The K721M-mutated acceptor TKD in Figure 11A maintains an inactive-like conformation despite retaining nearly all interactions with the donor that normally promote allosteric activation. This is evident from the slightly different orientations between the N- and C-lobes of each monomer in the structures shown in Figures 11A and 11B. The fact that EGFR$^{645-998}$(K721M) can form the asymmetric dimer shown in Figure 11 while adopting an inactive conformation suggests that the JM region plays a critical role in stabilizing this normally-activating mode of TKD association. An analogous situation can be inferred for ErbB4, where an inhibitor-bound inactive conformation crystallized as an asymmetric dimer (as in Figure 11) when part of the JM region was included in the protein (Wood, Shewchuk et al. 2008), but not when the JM region was absent (Qiu, Tarrant et al. 2008). Additionally, the requirement for the JM in stabilizing the asymmetric dimer is supported by biochemical evidence. The TKD alone is reported to exist mostly in a monomeric state at 150uM, while TKDs with intact JM regions exist predominantly as dimers, based on size exclusion experiments employing static light scattering (Jura, Endres et al. 2009).

Most of residues 664-671 (T669 is an exception) in the JM region of the acceptor (green) make contacts with the C-lobe of the donor (yellow), as shown in Figure 11C. Consistent with this, alanine substitution at these positions had a



Red Brewer et al. Mol Cell 34: 641-651 (2009)

Figure 11. The Acceptor JM Region 'Cradles' the C-lobe of the Donor in an Asymmetric TKD Dimer. A. Cartoon representation of the crystal structure of $EGFR^{645-998}$ harboring an inactivating K721M mutation. The TKD forms a crystallographic dimer that closely resembles the asymmetric TKD dimer seen for protein that lacks the complete JM region (Takishima, Griswold-Prenner et al. 1991; Zhang, Gureasko et al. 2006). The 'Acceptor' TKD is colored green, and 'Donor' TKD yellow. The N-lobe and C-lobe of donor and acceptor molecules are labeled, as are key helices in the donor C-lobe. Helix αC in the acceptor N-lobe is also marked. The N-terminal part of the JM region of the acceptor 'cradles' the donor C-lobe. The short N-terminal a-helix of the acceptor (residues 654-663) projects away from the donor, and makes no direct contacts. **B.** Structure of the active asymmetric dimer of the wild-type EGFR TKD lacking residues 645-671 from the JM region (PDB ID 2GS6), from Zhang et al. (2006). Features labeled in A are also labeled for this structure. **C.** Detailed view of side-chains in the acceptor JM region (green) that make contact with the C-lobe of the donor TKD. The orientation for this figure is shown in the cartoon representation in the inset, and the region shown is boxed. All side-chains present in the crystal structure from T654-R681 are shown. Those side-chains colored light grey could be replaced by alanine with no effect on ICD activity in Figure 1B (including T669). Side-chains colored green could not be replaced by alanine without significant loss of activity. Residue labels boxed in green correspond to the N-terminal half of the JM activation domain, where alanine substitutions have the greatest effect. Note that the N-terminal helix of the acceptor structure makes no direct contact with the donor. Structure by Sung Hee Choi, Diego Alvarado, Katarina Morevcevic, and Mark Lemmon.

strong inhibitory effect on EGFR ICD activation (Figure 2B). Residues 664- 671 of the acceptor JM region occlude 488Å^2 on the donor C-lobe surface.

Alanine substitutions at L664, V665, L668 or S671 are predicted to reduce van der Waal's interactions between the JM region and donor C-lobe (or hydrogen bonding interactions in the case of S671). Replacing E666 with alanine will abrogate electrostatic interactions with R949 in helix I of the donor C-lobe. Alanine substitution at P667, P670 or G672 could change local structure (and/or disrupt van der Waal's interactions) and thereby weaken JM interactions with the donor C-lobe. The data in Figure 2B show that each of these changes impairs EGFR ICD autophosphorylation as the structure generated by the Lemmon laboratory would predict. Of equal importance, where Figure 2B indicates no (or little) inhibitory effect of alanine substitution (T654-E663, and T669), Figure 11C shows that the side-chains in the JM region are not in direct contact with the donor C-lobe. These 'insensitive' side-chains are colored light gray in Figure 11C.

The α-helix at the N-terminus of the acceptor JM region (residues 653-663) makes no direct contact with the donor C-lobe. Its axis is approximately perpendicular to the C-lobe surface in Figures 11A and C. This helix is part of a reported binding site for calmodulin that encompasses residues 645-660 (Martin-Nieto and Villalobo 1998). The independence of this N-terminal helix in the structure presented here, and the disorder of the polybasic region that precedes it (residues 645-653), argue that the reported interactions of the EGFR JM region with calmodulin (Martin-Nieto and Villalobo 1998) and with negatively-charged

membrane surfaces (McLaughlin, Smith et al. 2005) might occur in concert with the cradling of the donor C-lobe seen here. Thus, proposed modes of JM-mediated EGFR regulation that involve these interactions (McLaughlin, Smith et al. 2005) may occur in parallel with the JM contributions to donor/acceptor interactions seen in Figure 11C.

These predictions are supported by structural modeling data from the Kuriyan group. In their model, dimerization of receptors results in the antiparallel association of JM-A (or exon 17) alpha helices. Jura et al. (2009) show, using molecular modeling, that the antiparallel alpha helices are created by residues 652-663 in the dimer state. These residues immediately precede the JMAD and demonstrate polarity whereby one 'face' of a donor helix is composed mainly of acidic residues which are thought to interact with the negatively charged plasma membrane, and the opposing face of an acceptor helix is composed of basic residues which face the kinase. Whether or not calmodulin recognizes this predicted anti-parallel helical structure is not known.

Phosphorylation of T654 and T669 were reported to provide mechanisms of negative feedback regulation of EGF receptor tyrosine kinase activity (Davis 1988; Heisermann, Wiley et al. 1990). Biochemical evidence supports roles for modification of these residues in the regulation of tyrosine kinase activity (Figure 5) (Thiel and Carpenter 2007). Structural explanations for this mode of negative feedback regulation are not as straightforward. Neither the T654 nor the T669 side-chain of the acceptor TKD is involved in direct interactions with the donor TKD, so substitution (or phosphorylation) does not reflect a loss of JM-

mediated interactions between TKD domains. In the case of T669 phosphorylation, the observed inhibition could result from changes in JM structure or stability when a phosphate group is added, globally disrupting JM-mediated interactions. Phosphorylation of T669 within the JMAD is predicted to disrupt its local structure, resulting in inhibition of EGFR activity by reducing the strength of acceptor/donor interactions. For T654 phosphorylation, the structure presented here does not suggest a clear possibility. Interestingly, alignment of EGFR (ErbB-1) and ErbB-3 JM sequences (Figure 2A) shows that the EGFR T669, which is conserved in both ErbB-2 and -4, is an aspartate in ErbB-3, consistent with reports that ErbB-3 has little or no kinase activity (Guy, Platko et al. 1994).

Examination of the interface between the JM region of the acceptor and C-lobe of the donor (Figure 11C) provides a possible structural basis for the EGFR activation by the V665M and L679F mutations found in NSCLC patients. The side-chain of V665 projects into a cavity on the surface of the C-lobe that is lined by aliphatic portions of Q788 and Y789 from helix E and Q825 from the β7/β8 loop (between the catalytic loop and activation loop of the TKD). The V665 side-chain does not fill this cavity (Figure 12A). However, substituting V665 with a methionine – as modeled by members of the Lemmon laboratory in the right-hand panel of Figure 12A – would fill the cavity completely, and is likely to stabilize the association of the acceptor's JM region with the donor C-lobe at this location. It is interesting to note that substitution of V665 with an alanine results in loss of tyrosine phosphorylation (Figure 2B and C). A second reported clinical

mutation, L679F, was also modeled. Mutation of L679 to phenylalanine could improve packing of the JM region with a PQPP sequence between helices αG and αH in the C-lobe of the donor (Figure 12B).

By solving the crystal structure of an EGFR TKD with its JM region intact, the Lemmon group showed that most of the 19-amino acid JMAD (residues 664-682) of an acceptor in the asymmetric TKD dimer makes contact with the C-lobe of the donor. Consistent with both biochemical and structural data, the most straightforward explanation for how the JMAD contributes to EGFR activation is that it stabilizes formation of the asymmetric dimer, thus promoting allosteric activation of the acceptor TKD. Supporting biochemical evidence shows that the addition of the JM results in a 67-fold increase in kinase activity in solution compared to wild-type ICD (Jura, Endres et al. 2009). This might reflect JMAD-stabilized formation of head-to-tail TKD polymers similar to those seen in crystals.

Identification of an important regulatory domain within the ICD but outside the TKD proper is of particular significance, because regions outside the kinase domain are rarely examined for mutations in clinical screens (Sharma, Bell et al. 2007). Where broader screens have been performed, a limited number of rare JM mutations in clinical tumor samples have been reported, but not characterized. I found that two of these previously reported clinical EGFR JM mutations (V665M and L679F) activate the receptor. L679F is tumor-specific, as the mutation was not found in normal tissue (Pallis, Voutsina et al. 2007). However, whether or not the V665M mutation is tumor-specific is not known. In

both cases, the degree of EGFR activation is similar to that seen for well-characterized oncogenic mutations (such as L834R) in the TKD itself (Sharma, Bell et al. 2007). Biochemical studies and structural arguments suggest that the V665M mutation stabilizes acceptor/donor interactions. The existence of such a clinically-observed mutation within the JMAD that I also show to be activating and transforming highlights the biological importance of this domain in regulating the EGF receptor kinase activity and cellular responsiveness. This activation domain is encoded by the same exon as a significant portion of the N-lobe of the TKD, and could be considered as a part of the TKD proper.

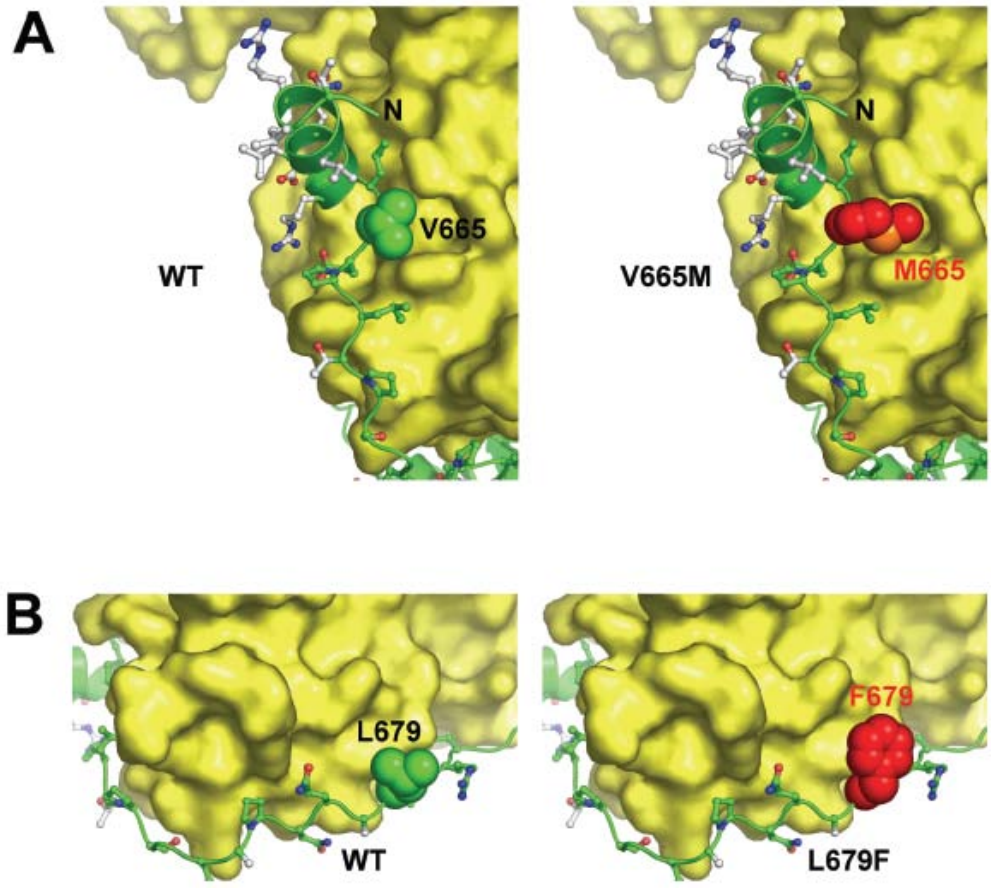


Figure 12. Mechanism of Activating JM Mutations. A. Contacts between the acceptor JM domain and donor C-lobe are shown with the V665 side-chain represented as space-filling spheres. The V665 side-chain fails to fill a cavity on the donor C-lobe formed by the side-chains of Q788, Y789 and Q825. However, as shown in the right-hand panel, a methionine (red) at position 665 – modeled for this figure - would completely fill the cavity, increasing van der Waal's contacts between acceptor and donor. **B.** Similarly, the L679F mutation is likely to promote interactions between the JM region (green) and the donor C-lobe, as indicated by modeling a phenylalanine at this position. Data by Sung Hee Choi, Diego Alvarado, Katarina Moravcevic, and Mark Lemmon

Red Brewer et al. Mol Cell 34: 641-651 (2009)

CHAPTER IV

THE p97 AAA-ATPASE IS REQUIRED FOR LIGAND-DEPENDENT NUCLEAR LOCALIZATION OF EPIDERMAL GROWTH FACTOR RECEPTOR

INTRODUCTION

Nuclear localization of the EGF receptor is known to mediate cellular effects such as induction of iNOS and cyclin D1 genes, which leads to increased cellular proliferation (Lala and Chakraborty 2001; Lin, Makino et al. 2001; Stacey 2003; Lo, Hsu et al. 2005). Evidence of nuclear localization of soluble EGF receptor exists as confocal and electron microscopy (EM) data, in both breast cancer cells and tumor tissue, as well as biochemical fractionation data (Lin, Makino et al. 2001; Lo, Hsu et al. 2005; Liao and Carpenter 2007). The confocal and EM data show diffuse EGF receptor localized to the nuclear compartment without evidence of nuclear membrane involvement, while the biochemical data demonstrates nuclear receptor solubility by extraction of full length receptor using a detergent-free high salt buffer. Nuclear full-length EGF receptor has been detected in numerous breast cancer cell lines, as well as in tissue samples from breast cancer patients (Lo, Xia et al. 2005). Since it is not detectable in the nuclei of normal tissue, this implies a role in breast cancer tumorigenesis. Also, nuclear EGF receptor in breast cancer tissue samples serves as a poor prognostic indicator compared to that of tissue in which EGF receptor is present, but not detected in the nucleus (Lo, Xia et al. 2005).

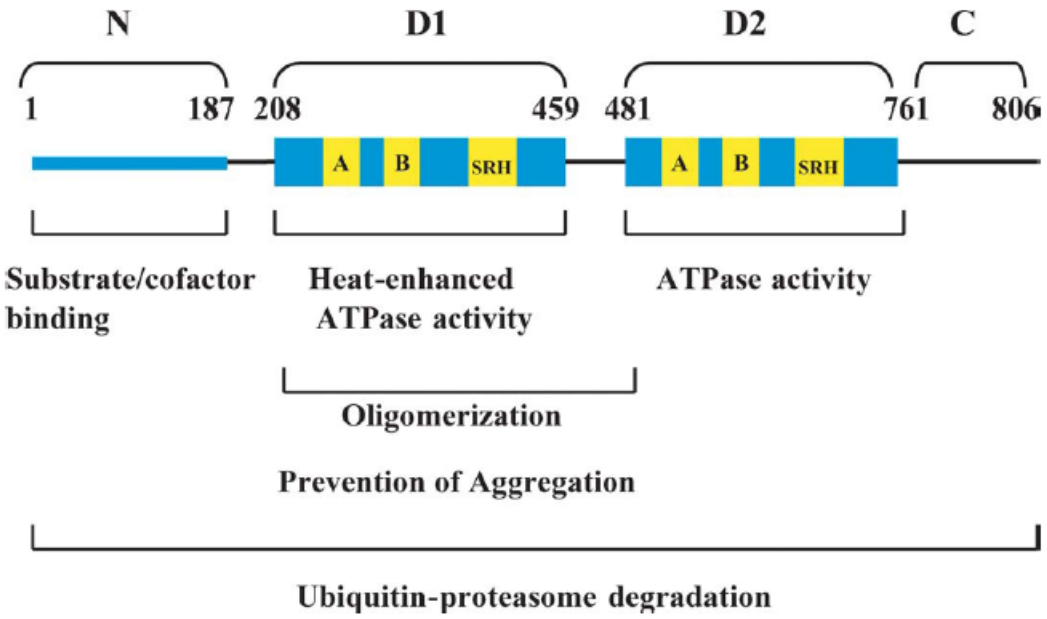
Trafficking of mature full-length EGF receptor to the nucleus has been shown to require the Sec61 translocon (Liao and Carpenter 2007). The Sec61 translocon is a trimeric complex consisting of α, β, and γ subunits(Osborne, Rapoport et al. 2005). It is localized to the endoplasmic reticulum (ER) and early Golgi (Greenfield and High 1999). The translocon exhibits bidirectional movement of proteins either from the cytosol into the ER, or by retro-translocation of misfolded proteins or toxins from the ER to the cytosol (Osborne, Rapoport et al. 2005). Nascent proteins are first translocated from the ribosome to the ER (through the translocon) to achieve proper conformation and glycosylation (Trombetta and Parodi 2003). In the ER, if proper folding is not achieved, proteins are then retro-translocated from the ER to the cytosol, where they degraded by the proteasome (Trombetta and Parodi 2003). The process of retro-translocation of misfolded proteins from the ER is known as ER-associated degradation (ERAD).

Data by Liao and Carpenter (2007) describe how the Sec61 translocon, an endoplasmic reticulum (ER) - localized protein complex, mediates trafficking of activated cell surface EGF receptor to the nucleus. Retrotranslocation of mature EGF receptor by the Sec61 translocon is thought to provide a means by which the receptor is liberated from its lipid membrane environment. Liao and Carpenter (2007) report that association of mature EGF receptor with the Sec61β subunit of the translocon is observed 3 hours post ligand addition, and that the nuclear receptor is high-salt extractable. Knockdown of the Sec61α subunit of the translocon results in loss of ligand-dependent nuclear localization of mature

EGF receptor. These results are consistent with a novel trafficking pathway in which the Sec61 translocon may function to free membrane-bound receptor to allow for trafficking of soluble receptor to the nucleus.

A central question in the Sec61-dependent mechanism of EGF receptor nuclear localization is how the Sec61-associated EGF receptor is 'extracted' or dislocated from the translocon. A candidate protein that may supply the motive force for this dislocation is the AAA-ATPase p97, since it is known to be required for the dislocation of substrates from the ER and derlin translocons (Ye, Meyer et al. 2001; Ye, Shibata et al. 2004).

P97, also known as valosin-containing protein (VCP) in mammalian systems and CDC48 in yeast, is a ubiquitous protein, comprising roughly 1% of the total cytosolic protein of a cell. It is a member of the type II AAA (ATPases associated with a variety of activities), and contains two AAA domains, also known as D1 and D2, which are responsible for nucleotide binding and hydrolysis (Wang, Song et al. 2004) (Figure 1). Hydrolysis of γ-phosphates from an ATP molecule bound to a functional homohexameric p97 is thought to supply mechanical force to the substrates of the ATPase in processes such as ERAD (Wang, Song et al. 2004). p97 participates in several different cellular processes in which its function appears to be governed by either its association with cofactors and/or ubiquitin chains of substrates. Cofactors of p97, which operate as targeting subunits, are quite variable and do not appear to fall into one discrete functional category. Rather, in concert with p97, its cofactors mediate a wide variety of cellular functions that range from DNA damage repair (BRCA1



Wang et al. *Journal of Structural Biology* 146: 44-57 (2004)

Figure 1. Schematic of p97 with Structural-Functional Relationships Indicated. The N-domain of p97 mediates interaction with protein substrates and cofactors. The D1 domain mediates the formation of stable hexamers of p97. The D2 domain binds and hydrolyses ATP. Both D-domains contain Walker A and B motifs and SRH (second region of homology). The Walker A and B motifs are traditionally described to mediate nucleotide-binding and hydrolysis, respectively. However, functional Walker motifs have only be described for the D2 domain. Likewise, a role for the SRH has been postulated in ATP hydrolysis. The C-domain is tyrosine phosphorylated.

and Wrn) and protein degradation (Ufd2 and Ufd3) to membrane trafficking (clathrin and syntaxin 1)(Dreveny, Pye et al. 2004). With the exception of one cofactor, Ufd3, all known p97 cofactors are known to associate with the N-domain of the ATPase (Zhao, Zhou et al. 2007).

Besides functional regulation due to cofactor association, posttranslational modifications can also affect p97 function. The prevalence of ubiquitin moieties on substrate proteins as well as the capacity of cofactors to mediate ubiquitin-dependent processes, suggests that besides functioning as a protein trafficking chaperone, p97 can also orchestrate ubiquitin-dependent processes (Ye 2006). When not associated with a cofactor, p97 can weakly associate with polyubiquitin moieties through a ubiquitin binding site in its N-domain. However, p97 is known to associate with cofactors that contain ubiquitin binding domains (such as Npl4 and p47) as well as E3 ligases (such as Hrd1 and Gp78)(Ye 2006).

Although p97 exhibits nucleo-cytoplasmic distribution, its yeast homologue Cdc48, is reported to localize to the cytosolic leaflet of the ER or the nucleus based on its tyrosine phosphorylation (Latterich, Frohlich et al. 1995; Madeo, Schlauer et al. 1998). p97 was first reported to be tyrosine phosphorylated in response to T-cell receptor activation (Egerton, Ashe et al. 1992). It has since been reported to be phosphorylated by Src as well as by activated Akt in response to phorbol ester, FGF, or EGF (Vandermoere, El Yazidi-Belkoura et al. 2006; Li, Zhao et al. 2008). Tyrosine phosphorylation of the penultimate Tyr 805 in the C-domain is known to result in redistribution of the yeast homologue Cdc48 to the nuclear compartment, suggesting a regulatory role for this modification in

subcellular localization (Madeo, Schlauer et al. 1998). Moreover, tyrosine phosphorylation of this residue is reported to abolish the ERAD-associated activity of p97 (Zhao, Zhou et al. 2007; Li, Zhao et al. 2008), adding yet another layer of regulatory complexity to the function of the protein. In addition, mass spectrometric analysis of p97 shows it to be acetylated on lysine residues within the D1 and D2 domains, and mutational analysis has demonstrated a role for modification of these residues in influencing ATP binding affinity as well as catalysis(Mori-Konya, Kato et al. 2009), which ultimately controls the mechanical 'ratcheting' function of p97.

In this chapter, I describe a role for the p97 AAA-ATPase in nuclear trafficking of the EGF receptor using coprecipitation and knockdown assays.

RESULTS

P97 Associates with EGF Receptor in an EGF-Dependent Manner

In order to determine whether p97 may play a role in the Sec61-dependent EGF receptor nuclear localization, a coprecipitation assay was performed which included time points consistent with maximal ligand-dependent Sec61-EGF receptor association as previously reported by Liao and Carpenter (Liao and Carpenter 2007). The top panel of Figure 2 shows that 3 hours post-ligand addition, endogenous p97 maximally associates with endogenous EGF receptor in MDA-MB-468 cells. This finding is consistent with the time course

reported for maximal Sec61-EGFR association (Liao and Carpenter 2007). Additionally, p97 is shown to be minimally tyrosine phosphorylated at 3 hours post-ligand addition (Figure 2, bottom panel), suggesting it may be localized to the cytosolic compartment of the cell, based on studies in the yeast homologue Cdc48 (Madeo, Schlauer et al. 1998).

Knockdown of p97 Results in Decreased Ligand-Dependent EGF Receptor Nuclear Localization

If p97 is required to dislocate the EGF receptor from the translocon, the outcome of which is nuclear trafficking, then transient knockdown of p97 should result in reduction of ligand-induced nuclear EGF receptor. Three shRNA clones were tested independently for knockdown of endogenous p97 in HEK293 cells (Figure 3A). The non-silencing control shRNA (demonstrated to have no effect on gene silencing) and clone 111071 showed no significant reduction in p97 protein levels, however, the expression of clone 224206 resulted in a significant reduction of p97 protein levels. Specificity of the p97 knockdown is demonstrated by the maintenance of tubulin levels as shown in Figure 3A. In Figure 3B, the nuclear fraction prepared from cells expressing both EGF receptor and the p97 shRNA clone 224206 shows a substantial reduction in the amount of ligand-dependent EGF receptor compared to the nuclear fraction of cells expressing EGF receptor only. Additionally, no substantial change in EGF receptor levels is observed in non-nuclear fractions prepared from cells

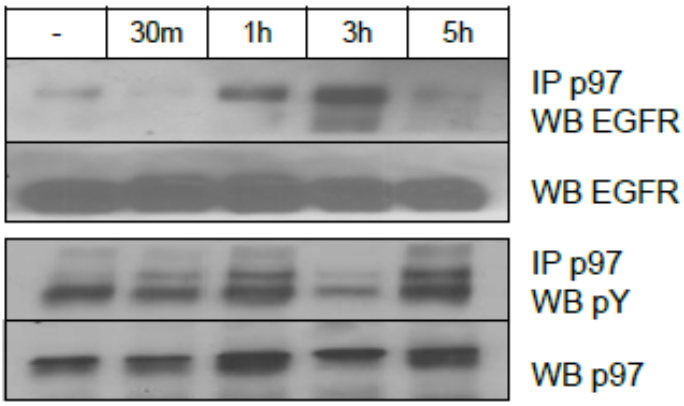


Figure 2. p97 Associates with EGF Receptor.
MDA-MB-468 cells were serum-starved and treated with EGF for the indicated times. Cell lysates were immunoprecipitated with anti-p97. Immunoprecipitates and lysates were subjected to SDS-PAGE and membranes were western blotted with anti-EGFR, anti-pY, or anti-p97. P97 maximally associates with EGF receptor and is minimally tyrosine phosphorylated at 3 hours post-EGF.

Red Brewer et al. Unpublished data (2009)

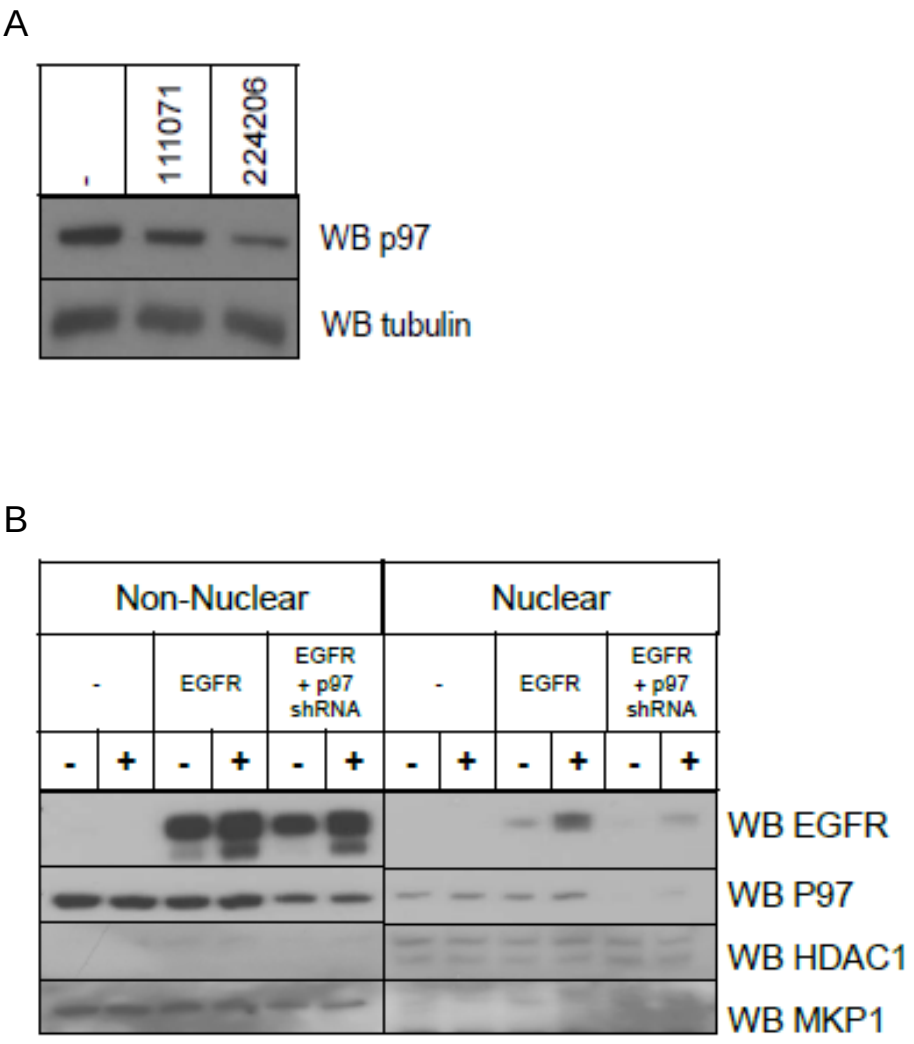


Figure 3. p97 Knockdown Reduces Ligand-Dependent Nuclear EGF Receptor. A) HEK293 cells were transiently transfected with the p97 shRNA construct clones indicated and cultured for 72 hours. Cells were lysed and lysates subjected to SDS-PAGE. Membranes were western blotted with anti-p97 and anti-tubulin. **B)** HEK293 cells ectopically expressing flag-EGFR with or without a p97shRNA construct as indicated were cultured for 60 hours and serum-starved for 12 hours. Cells were then treated or not with EGF for 3.5 hours. Cells were fractioned into nuclear and non-nuclear lysates. Lysates were subjected to SDS-PAGE. Membranes were western blotted with anti-EGFR, anti-p97, anti-HDAC1 (as a nuclear marker), and anti-Mkp1 (as a cytosolic marker). Knockdown of p97 results in a significant decrease in ligand-dependent nuclear EGF receptor.

Red Brewer et al. Unpublished data (2009)

transiently expressing EGF receptor compared to fractions prepared from cells transiently expressing EGF receptor and p97 shRNA. This result indicates that p97 is required for ligand-dependent nuclear trafficking of EGF receptor.

p97 Preferentially Associates with a Constitutively Active EGF Receptor JM Mutant

Given the results in Figure1, that show that EGF receptor association with p97 is a ligand-dependent process, I asked whether the tyrosine kinase activity of the receptor is required for association with p97. Cells expressing either wild-type receptor, the constitutively active mutants V665M or L834R, or the kinase-dead mutant K721R, were tested for the capacity to associate with p97 at the previously determined time point of maximal association, i.e. 3 hours following the addition of EGF. Figure 4 shows that not only does the constitutively active V665M mutant receptor associate with p97 more than wild-type receptor, but that this increased association remains a ligand-dependent process. Surprisingly, the constitutively active mutant receptor L834R does not associate with p97 in the absence or presence of ligand at the given time point. These results suggest that ligand-dependent trafficking of the receptor is required and that p97 may possibly recognize specific receptor sequences or conformations.

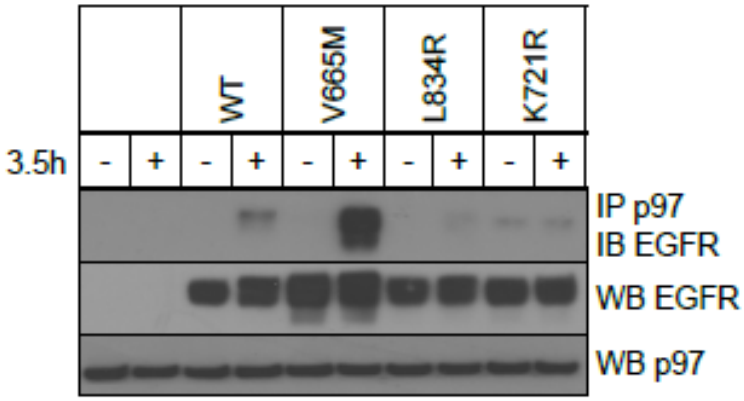


Figure 4. Ligand-dependent EGFR:p97 Association is Augmented by Expression of the Constitutively Active EGFR JM Mutant V665M. HEK293 cells ectopically expressing the indicated full-length EGFR receptor cDNA constructs were serum-starved and either treated with EGF for 3.5 hours or not. Lysates were immunoprecipitated with anti-p97. Immunoprecipitates and lysates were subjected to SDS-PAGE and membranes were western blotted with anti-EGFR or anti-p97. EGFR-V665M expressing 293 cells exhibit more ligand-dependent p97 association than compared to WT.

Red Brewer et al. Unpublished data (2009)

The EGF Receptor Mutant V665M Exhibits Increased Nuclear Localization Compared to Wild-Type Receptor

Due to the increased association of p97 with the EGF receptor mutant V665M compared to wild-type receptor (Figure 4), as well as the requirement of p97 for trafficking of the receptor to the nucleus (Figure 3), I hypothesized that this mutant receptor is likely trafficked to the nucleus more efficiently than wild-type receptor. Indeed, as shown in Figure 5, western blotting of the nuclear fractions of cells expressing either wild-type or the V665M mutant demonstrates a significant increase in the levels of mutant EGF receptor in the nuclear fraction compared to wild-type.

DISCUSSION

The nuclear localization of several transmembrane protein ICD fragments, such as amyloid precursor protein, Notch, and ErbB-4, have been shown to be ligand-dependent processes that result in transcriptional effects in cells and mouse models. The existence of cleavage fragments and the identification of proteases required for their formation has resulted in a description of the phenomenon known as regulated intramembrane proteolysis (RIP), and has served as the prototypical mechanism required for nuclear localization of membrane protein fragments.

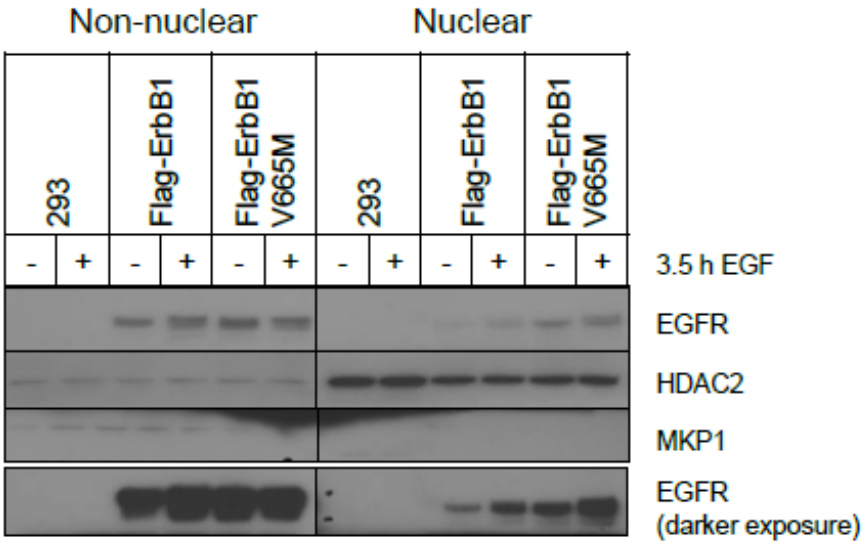


Figure 5. Ligand-dependent Nuclear Localization of EGFR-V665M is Greater than WT Receptor. HEK293 cells ectopically expressing either full-length EGF receptor (WT) or the activating JM mutant V665M were serum-starved and treated or not with EGF for 3.5 hours. Cells were fractioned into nuclear and non-nuclear lysates. Lysates were subjected to SDS-PAGE and western blotted with anti-EGFR, HDAC2 (as a nuclear marker), or MKP1 (as a cytosolic marker). More full-length EGF receptor containing the V665M mutation is shown to localize to the nucleus compared to WT.

Red Brewer et al. Unpublished data (2009)

In contrast to RIP, it has been shown more recently that intact EGF receptor is localized to the nucleus (Lin, Makino et al. 2001; Liao and Carpenter 2007). The presence of intact EGF receptor in the nucleus is coincident with upregulation of genes whose protein products can mediate proliferative effects. The mechanism for localization of such a large, transmembrane protein to the nucleus has led to the discovery of a novel means of receptor trafficking. The Sec61-dependent trafficking of the EGF receptor provides a process in which the protein may be liberated from its lipid bilayer environment(Liao and Carpenter 2007). Two proteins, heat shock protein 70 and importin-beta, are reported to be required for trafficking of the intact receptor to the nuclear compartment(Liao and Carpenter 2007). Data presented here (Figures 2 and 3) are consistent with the requirement of the chaperone p97 in the process of nuclear translocation of EGF receptor.

Data presented in Figure 4 suggests that active tyrosine kinase domain conformations may be required for maximal recognition by p97. Another possibility for the differences seen in V665M versus wild-type receptor association with p97 may be linked to differences in receptor ubiquitination. Upon activation, it is reported that receptor ubiquitination occurs in a site-specific manner via the ubiquitin ligase Cbl (Levkowitz, Waterman et al. 1998). Additionally, reports have shown that the activated receptor contains both mono- and poly-ubiquitin moieties, and mutation of multiple ubiquitin-conjugation sites can result in impaired receptor internalization (Huang, Kirkpatrick et al. 2006). Given the propensity of p97, and many of its cofactors, for association with

ubiquitinated substrates, it is plausible that subpopulations of tyrosine phosphorylated and ubiquitinated receptor may be preferential p97 substrates. The addition of multiple post-translational modifications may provide a mechanism of tightly regulating the trafficking of a relatively small percentage of EGF receptor that is localized to the nucleus.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation, I have shown that a specific portion of the juxtamembrane sequence of the EGF receptor is required for its activation, and thus functions as an activation domain. Data presented in Chapter 3 (Figures 2, 3, and 5) illustrate that exon 18-encoded residues of the JM region constitute a JMAD and furthermore, that these residues stabilize an active conformation of the receptor (Red Brewer, Choi et al. 2009). This is the first description of a juxtamembrane sequence of any RTK acting in an activating, rather than inhibitory, manner (Hubbard 2004). Additional data support the mechanism by which a clinically reported JM region mutation effects activation of the receptor leading to a transformed phenotype in cell culture and tumorigenesis in athymic nude mice (Chapter 3, Figures 6, 7, and 9). This is the first mutation, outside of the tyrosine kinase domain proper, shown to be activating and oncogenic and establishes the biological significance of the JMAD.

These data provide insight into the allosteric requirements for activation of the tyrosine kinase, and thus indicate a potential new target for inhibition of the kinase by disruption of JMAD residue contacts in the asymmetric dimer. Figure 10 of Chapter 3 illustrates the insensitivity of a clinically reported JM mutation to the approved EGF receptor-directed chemotherapeutic ATP-mimetic erlotinib. The data imply that small molecule inhibitors directed at sites other than the

nucleotide binding pocket of the receptor may be effective inhibitors, and would inherently have fewer off-target side effects.

To extend the research presented in this dissertation, I propose using peptide inhibitors of the EGF receptor asymmetric dimer interface that include residues of the JMAD. Proof of concept for this mode of allosteric inhibition is supplied by the EGF-responsive protein Mig-6. Mig-6 is reported to associate with ErbB-1 and -2 and result in their inactivation (Hackel, Gishizky et al. 2001; Anastasi, Sala et al. 2005; Zhang, Pickin et al. 2007). Short Mig-6 peptides have been shown to associate with and inhibit dimer formation of ErbB-1 by preventing allosteric contact of tyrosine kinase domain monomers (Zhang, Pickin et al. 2007)(Table 1).

Peptide inhibitors used in such a study would include mimics of the JMAD as well as the positive control Mig-6 peptide published by the Kuriyan group (Zhang, Pickin et al. 2007)(Table 2). Presumably, a JMAD peptide would have a lower affinity for binding to the kinase than a Mig-6 peptide, since Mig-6 associates with activated receptors to bring about their inactivation, thus competing for binding to the same allosteric interface as the JMAD. A central question is whether or not incorporation of a methionine to fill the aliphatic pocket created by the donor monomer side-chains of Q788, Y789 and Q825 would result in increased peptide binding affinity and/or kinase inhibition, since methionine substitution at Val 665 in the JMAD is proposed to constitutively activate the kinase by stabilizing the active dimer (Red Brewer, Choi et al. 2009). I predict that a chimeric Mig-6/JMAD peptide with a methionine substitution at the

position that fills the aliphatic pocket created by the side-chains of Q788, Y789 and Q825 will result in a more effective peptide (higher affinity/increased kinase inhibition) than the use of JMAD-only or Mig-6-only peptides (Table 2). As with the Mig-6 study by the Kuriyan group (2008), fluorescently labeled peptides could be tested for association with purified EGF receptor intracellular domain (ICD) protein. A range of concentrations could be tested for association to the ICD protein. Fluorescence polarization experiments would provide data regarding the concentrations of peptide(s) required for maximal association of peptide to ICD, bearing in mind that the beginning target range for any small molecule inhibitor is the 10uM range. Additionally, analytical ultracentrifugation experiments could be conducted to assess the concentration of peptide required to competitively inhibit dimer formation. Using these two methods, both association and inhibition can be tested.

The successful identification of ErbB inhibitory peptides could be used as proof of concept for the development of small molecule inhibitor(s). Identification of an allosteric inhibitor has been shown to be effective towards Bcr-Abl (Adrian, Ding et al. 2006). Production of an ErbB-specific allosteric inhibitor would presumably target multiple ICD mutations, such as the NSCLC L834R and the gatekeeper T776M by preventing dimerization of kinase domain monomers and thus, preventing autophosphorylation. The gatekeeper mutation T776M has been described as insensitive to gefitinib. In this case, mutation results in increased ATP affinity as well as steric hindrance of the ATP analog gefitinib (Mulloy, Ferrand et al. 2007; Yun, Boggon et al. 2007). Even in cases where

B	I	Name	Sequence
+	+	60-residue	(GPLGS)^{315}RPPKVPPREPLSPSNSRTPSPKSLPSYLNGVMPPTQSFAPDPKYVSSKALQRQNSEGSAS^{374}
+	+	40-residue	Ac-^{325}LSPSNSRTPSPKSLPSYLNGVMPPTQSFAPDPKYVSSKAL^{364}
-	-	25-residue	Ac-^{340}SYLNGVMPPTQSFAPDPKYVSSKAL^{364}
+	+	30-residue	Ac-^{334}SPKSLPSYLNGVMPPTQSFAPDPKYVSSKA^{363}
+	+	30-residueFl	Fl-Ahx-^{334}SPKSLPSYLNGVMPPTQSFAPDPKYVSSKA^{363}
+	+	336-412	^{336}KSLPSYLNGVLPPTQSFAPDPKYVSSKAL- -QRQNSEGSASKVPCILPIIENGKKVSSTHYYLLPERPPYLDKYEKFFR^{412}
-	-	336-412 (Y358A)	^{336}KSLPSYLNGVLPPTQSFAPDPKAVSSKAL- -QRQNSEGSASKVPCILPIIENGKKVSSTHYYLLPERPPYLDKYEKFFR^{412}

(): residues from the expression vector; Ac: acetyl group; Fl: fluorescein; Ahx, aminohexanoic acid; Red: segment 1; Blue: mutations

Adapted from Zhang et al. Nature 450: 741-745 (2007)

Table 1. Reported Mig6 Peptides that Exhibit Either Binding (B) and/or Inhibition (I) of EGF Receptor Kinase Activity. Peptides comprised of Mig6 sequence that binds in the allosteric dimer interface ('segment 1' in red) as well as with the additional sequence were tested for both binding (B) to EGFR kinase and inhibition (I) of kinase activity using anisotropy and vesicle-based activity assays. The entire segment 1 sequence (in red) is required for binding and inhibition of any of the peptides to the WT kinase, while the capacity of the peptides to inhibit the L834R mutant requires both segments 1 and 2 (336-412).

Peptide	Sequence
Mig6 60 res.	^{325}RPPKVPPREPLSPSNSRTPSPKSLPSYLNGVMPPTQSFAPDPKYVSSKALQRQNSEGSAS^{374}
JMAD	^{664}LVEPLTPSGEAPNQALLRI^{682}
JMAD/Mig6	LVEPLTPSGEAPNQALSLPSYLNGVMPPTQSFAPDPKYVSSKALQRQNSEGSAS
JMAD-VM/Mig6	LMEPLTPSGEAPNQALSLPSYLNGVMPPTQSFAPDPKYVSSKALQRQNSEGSAS

Table 2. Proposed Peptides for Binding and Inhibition of ErbB-1 and-2 Kinases. A control Mig6 60 residue peptide will be used as a control, since it has been reported to result in maximum binding and inhibition of the ErbB-1 kinase (Zhang, Pickin et al. 2007). Additional peptides include the JMAD-only, a JMAD/Mig6 chimera, and a JMAD V665M/Mig6 chimera.

there is an observed increase of ATP affinity for the kinase, a drug targeting a prerequisite activation step would hypothetically be effective.

Within this dissertation, I have also shown that p97 associates with EGF receptor in an EGF-dependent manner at a time point consistent with the maximum association of Sec61 with the receptor, and that knockdown of p97 leads to a significant reduction in nuclear EGF receptor (Chapter IV, Figures 2 and 3). The initial hypothesis that p97 functions in dislocation of the receptor from the translocon was not testable by the *in vitro* retrotranslocation assay using crude microsome fractions. In order to place p97 in the Sec61-dependent nuclear EGF receptor pathway described by Liao and Carpenter, different experimental techniques may be employed (Liao and Carpenter 2007). For instance, further purification of cellular fractions (beyond that of crude microsomes) may be necessary to show biochemical evidence of p97 involvement in the retrotranslocation process. Purified ER fractions have been used to demonstrate the requirement of a functional Sec61 complex as well as Hsp70 in the Sec61-dependent pathway (Liao and Carpenter 2007).

Chemical inhibition of ERAD has been demonstrated with the use of eeyarestatin (Wang, Li et al. 2008), which is reported to associate with p97 and inhibit the p97-Ufd1-p47 dislocation of substrates from the ER, resulting in an accumulation of substrates, such as Ataxin-3, that are normally trafficked in a p97-dependent manner to the proteasome for destruction (Wang, Li et al. 2008). Use of this compound may provide data that is consistent with the function of p97 at the ER.

Another unanswered question is how p97 associates with the EGF receptor. Since p97 is known to associate with substrates though cofactors and/or ubiquitination, identification of potential cofactors or modification by ubiquitin in the Sec61-dependent nuclear localization process would be informative. Figure 4 of Chapter IV demonstrates that the EGF receptor mutant V665M associates with p97 more than wild-type receptor at 3.5 hours post-ligand addition. This mutant provides a tool to test the hypothesis that ubiquitin may play a role in mediating p97 association. I hypothesize that the mutant receptor will either show an increase in total ubiquitin, or an increase in a different type of ubiquitin linkage(i.e. K48-, K63- linked Ub), compared to wild-type receptor.

Mapping of the EGF receptor-p97 interaction site may also delineate a mode for increased association and nuclear trafficking of the mutant receptor (chapter IV, Figures 4 and 5). A common feature of three p97 interacting proteins, Ataxin-3, slow Wallerian degeneration protein, and Hrd1, is the requirement of a short N-terminal polybasic sequence. Truncation of the N-terminal portions of these substrate proteins results in loss of p97 association (Boeddrich, Gaumer et al. 2006; Wilbrey, Haley et al. 2008). Certainly, such a polybasic sequence exists within the juxtamembrane sequence of the EGF receptor and comprises a tripartite NLS(Hsu and Hung 2007). Examination of a potential role of the EGF receptor JM-localized polybasic sequence in ligand-dependent association with p97 is a plausible experimental direction. Generation of purified p97 protein to test association with transiently expressed EGF receptor mutants would likely yield information about the required receptor

sequence as well as protein(s) in complex with p97 that may be required for association with EGF receptor. It is also possible that the V665M receptor preferentially associates with a p97 cofactor that is responsible for nuclear trafficking of substrates.

BIBLIOGRAPHY

Abe, Y., M. Odaka, et al. (1998). "Disulfide bond structure of human epidermal growth factor receptor." J Biol Chem **273**(18): 11150-7.

Adrian, F. J., Q. Ding, et al. (2006). "Allosteric inhibitors of Bcr-abl-dependent cell proliferation." Nat Chem Biol **2**(2): 95-102.

Anastasi, S., G. Sala, et al. (2005). "Loss of RALT/MIG-6 expression in ERBB2-amplified breast carcinomas enhances ErbB-2 oncogenic potency and favors resistance to Herceptin." Oncogene **24**(28): 4540-8.

Balak, M. N., Y. Gong, et al. (2006). "Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors." Clin Cancer Res **12**(21): 6494-501.

Binns, K. L., P. P. Taylor, et al. (2000). "Phosphorylation of Tyrosine Residues in the Kinase Domain and Juxtamembrane Region Regulates the Biological and Catalytic Activities of Eph Receptors." Mol. Cell. Biol. **20**: 4791-4805.

Biscardi, J. S., M.-C. Maa, et al. (1999). "c-Src-mediated Phosphorylation of the Epidermal Growth Factor Receptor on Tyr845 and Tyr1101 Is Associated with Modulation of Receptor Function." J. Biol. Chem. **274**(12): 8335-8343.

Boeddrich, A., S. Gaumer, et al. (2006). "An arginine/lysine-rich motif is crucial for VCP/p97-mediated modulation of ataxin-3 fibrillogenesis." EMBO J **25**(7): 1547-58.

Boerner, J. L., M. L. Demory, et al. (2004). "Phosphorylation of Y845 on the epidermal growth factor receptor mediates binding to the mitochondrial protein cytochrome c oxidase subunit II." Mol Cell Biol **24**(16): 7059-71.

Buhrow, S. A., S. Cohen, et al. (1983). "Characterization of the interaction of 5'-p-fluorosulfonylbenzoyl adenosine with the epidermal growth factor receptor/protein kinase in A431 cell membranes." J Biol Chem **258**(12): 7824-7.

Buhrow, S. A., S. Cohen, et al. (1982). "Affinity labeling of the protein kinase associated with the epidermal growth factor receptor in membrane vesicles from A431 cells." J Biol Chem **257**(8): 4019-22.

Carpenter, G. (1979). "Solubilization of membrane receptor for epidermal growth factor." Life Sci **24**(18): 1691-7.

Chan, P. M., S. Ilangumaran, et al. (2003). "Autoinhibition of the Kit receptor tyrosine kinase by the cytosolic juxtamembrane region." Mol. Cell. Biol. **23**: 3067-3078.

Chen, W. S., C. S. Lazar, et al. (1987). "Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor." Nature **328**(6133): 820-3.

Chen, X., Y. Su, et al. (2005). "Increased plasma MMP9 in integrin alpha1-null mice enhances lung metastasis of colon carcinoma cells." Int. J. Cancer **116**: 52-61.

Cho, H. S. and D. J. Leahy (2002). "Structure of the extracellular region of HER3 reveals an interdomain tether." Science **297**(5585): 1330-3.

Choi, S. H., J. M. Mendrola, et al. (2007). "EGF-independent activation of cell-surface EGF receptors harboring mutations found in gefitinib-sensitive lung cancer." Oncogene **26**: 1567-1576

Chou, T. Y., C. H. Chiu, et al. (2005). "Mutation in the tyrosine kinase domain of epidermal growth factor receptor is a predictive and prognostic factor for gefitinib treatment in patients with non-small cell lung cancer." Clin. Cancer Res. **11**: 3750-3757.

Cohen, S. (1960). "Purification of a Nerve-Growth Promoting Protein from the Mouse Salivary Gland and Its Neuro-Cytotoxic Antiserum." Proc Natl Acad Sci U S A **46**(3): 302-11.

Cohen, S. (1962). "Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal." J Biol Chem **237**: 1555-62.

Cohen, S., H. Ushiro, et al. (1982). "A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles." J Biol Chem **257**(3): 1523-31.

Davis, R. J. (1988). "Independent mechanisms account for the regulation by protein kinase C of the epidermal growth factor receptor affinity and tyrosine-protein kinase activity." J Biol Chem **263**(19): 9462-9.

Downward, J., M. D. Waterfield, et al. (1985). "Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. Effect on tyrosine kinase activity and ligand binding affinity." J. Biol. Chem. **260**(27): 14538-14546.

Downward, J., Y. Yarden, et al. (1984). "Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences." Nature **307**(5951): 521-7.

Dreveny, I., V. E. Pye, et al. (2004). "p97 and close encounters of every kind: a brief review." Biochem Soc Trans **32**(Pt 5): 715-20.

Egerton, M., O. R. Ashe, et al. (1992). "VCP, the mammalian homolog of cdc48, is tyrosine phosphorylated in response to T cell antigen receptor activation." Embo J **11**(10): 3533-40.

Elleman, T. C., T. Domagala, et al. (2001). "Identification of a determinant of epidermal growth factor receptor ligand-binding specificity using a truncated, high-affinity form of the ectodomain." Biochemistry **40**(30): 8930-9.

Ferguson, K. M., M. B. Berger, et al. (2003). "EGF Activates Its Receptor by Removing Interactions that Autoinhibit Ectodomain Dimerization." Molecular Cell **11**(2): 507.

Frederick, L., X. Y. Wang, et al. (2000). "Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas." Cancer Res **60**(5): 1383-7.

Gao, S. P., K. G. Mark, et al. (2007). "Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas." J Clin Invest **117**(12): 3846-56.

Garrett, T. P., N. M. McKern, et al. (2002). "Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha." Cell **110**(6): 763-73.

Greenfield, J. J. and S. High (1999). "The Sec61 complex is located in both the ER and the ER-Golgi intermediate compartment." J Cell Sci **112 (Pt 10)**: 1477-86.

Griffith, J., J. Black, et al. (2004). "The structural basis for autoinhibition of FLT3 by the juxtamembrane domain." Mol. Cell **13**: 169-178.

Guy, P. M., J. V. Platko, et al. (1994). "Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity." Proc Natl Acad Sci U S A **91**(17): 8132-6.

Guy, P. M., J. V. Platko, et al. (1994). "Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity." Proc. Natl. Acad. Sci. U. S. A. **91**: 8132-8136.

Hackel, P. O., M. Gishizky, et al. (2001). "Mig-6 is a negative regulator of the epidermal growth factor receptor signal." Biol Chem **382**(12): 1649-62.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hanks, S. K. and T. Hunter (1995). "Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification." FASEB J **9**(8): 576-96.

Haura, E. B., Z. Zheng, et al. (2005). "Activated epidermal growth factor receptor-Stat-3 signaling promotes tumor survival in vivo in non-small cell lung cancer." Clin Cancer Res **11**(23): 8288-94.

He, C., M. Hobert, et al. (2002). "The epidermal growth factor receptor juxtamembrane domain has multiple basolateral plasma membrane localization determinants, including a dominant signal with a polyproline core." J. Biol. Chem. **277**(38284-38293).

He, C., M. Hobert, et al. (2002). "The epidermal growth factor receptor juxtamembrane domain has multiple basolateral plasma membrane localization determinants, including a dominant signal with a polyproline core." J. Biol. Chem. **277**(41): 38284-38293.

Heisermann, G. J., H. S. Wiley, et al. (1990). "Mutational removal of the Thr669 and Ser671 phosphorylation sites alters substrate specificity and ligand-induced internalization of the epidermal growth factor receptor." J. Biol. Chem. **265**(22): 12820-12827.

Holbro, T., G. Civenni, et al. (2003). "The ErbB receptors and their role in cancer progression." Exp Cell Res **284**(1): 99-110.

Honegger, A. M., R. M. Kris, et al. (1989). "Evidence that Autophosphorylation of Solubilized Receptors for Epidermal Growth Factor is Mediated by Intermolecular Cross-Phosphorylation." PNAS **86**(3): 925-929.

Honegger, A. M., A. Schmidt, et al. (1990). "Evidence for epidermal growth factor (EGF)-induced intermolecular autophosphorylation of the EGF receptors in living cells." Mol. Cell. Biol. **10**(8): 4035-4044.

Hsu, S.-C. and M.-C. Hung (2007). "Characterization of a Novel Tripartite Nuclear Localization Sequence in the EGFR Family." J. Biol. Chem. **282**(14): 10432-10440.

Hsu, S. C. and M. C. Hung (2007). "Characterization of a novel tripartite nuclear localization sequence in the EGFR family." J Biol Chem **282**(14): 10432-40.

Huang, F., D. Kirkpatrick, et al. (2006). "Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain." Mol Cell **21**(6): 737-48.

Hubbard, S. R. (2004). "Juxtamembrane autoinhibition in receptor tyrosine kinases." Nat. Rev. Mol. Cell Biol. **5**: 464-471.

Hubbard, S. R. (2004). "Juxtamembrane autoinhibition in receptor tyrosine kinases." Nat Rev Mol Cell Biol **5**(6): 464-71.

Jiang, J., H. Greulich, et al. (2005). "Epidermal growth factor-independent transformation of Ba/F3 cells with cancer-derived epidermal growth factor receptor mutants induces gefitinib-sensitive cell cycle progression." Cancer Res. **65**: 8968–8974.

Jura, N., N. F. Endres, et al. (2009). "Mechanism for activation of the EGF receptor catalytic domain by the juxtamembrane segment." Cell **137**(7): 1293-307.

Kazazic, M., V. Bertelsen, et al. (2009). "Epsin 1 is involved in recruitment of ubiquitinated EGF receptors into clathrin-coated pits." Traffic **10**(2): 235-45.

Kil, S. J., M. Hobert, et al. (1999). "A leucine-based determinant in the epidermal growth factor receptor juxtamembrane domain is required for the efficient transport of ligand-receptor complexes to lysosomes." J Biol Chem **274**(5): 3141-50.

Kil, S. J., M. Hobert, et al. (1999). "A leucine-based determinant in the epidermal growth factor receptor juxtamembrane domain is required for the efficient transport of ligand-receptor complexes to lysosomes." J. Biol. Chem. **274**: 3141-3150.

Klapper, L. N., S. Glathe, et al. (1999). "The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors." Proc Natl Acad Sci U S A **96**(9): 4995-5000.

Kobayashi, S., T. J. Boggon, et al. (2005). "EGFR mutation and resistance of non-small-cell lung cancer to gefitinib." N Engl J Med **352**(8): 786-92.

Kosaka, T., Y. Yatabe, et al. (2006). "Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib." Clin Cancer Res **12**(19): 5764-9.

Lala, P. K. and C. Chakraborty (2001). "Role of nitric oxide in carcinogenesis and tumour progression." Lancet Oncol **2**(3): 149-56.

Latterich, M., K. U. Frohlich, et al. (1995). "Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes." Cell **82**(6): 885-93.

Lax, I., F. Bellot, et al. (1989). "Functional analysis of the ligand binding site of EGF-receptor utilizing chimeric chicken/human receptor molecules." EMBO J **8**(2): 421-7.

Levi-Montalcini, R. and S. Cohen (1956). "In Vitro and in Vivo Effects of a Nerve Growth-Stimulating Agent Isolated from Snake Venom." Proc Natl Acad Sci U S A **42**(9): 695-9.

Levkowitz, G., H. Waterman, et al. (1998). "c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor." Genes Dev. **12**(23): 3663-3674.

Li, G., G. Zhao, et al. (2008). "Tyrosine phosphorylation of ATPase p97 regulates its activity during ERAD." Biochem Biophys Res Commun **375**(2): 247-51.

Liao, H.-J. and G. Carpenter (2007). "Role of the Sec61 Translocon in EGF Receptor Trafficking to the Nucleus and Gene Expression." Mol. Biol. Cell **18**(3): 1064-1072.

Liao, H. J. and G. Carpenter (2007). "Role of the Sec61 translocon in EGF receptor trafficking to the nucleus and gene expression." Mol Biol Cell **18**(3): 1064-72.

Lin, S.-Y., K. Makino, et al. (2001). "Nuclear localization of EGF receptor and its potential new role as a transcription factor." Nat. Cell Biol. **3**: 802-808.

Lin, S.-Y., K. Makino, et al. (2001). "Nuclear localization of EGF receptor and its potential new role as a transcription factor." Nat Cell Biol **3**(9): 802.

Lin, S. Y., K. Makino, et al. (2001). "Nuclear localization of EGF receptor and its potential new role as a transcription factor." Nat Cell Biol **3**(9): 802-8.

Linggi, B. and G. Carpenter (2006). "ErbB receptors: new insights on mechanisms and biology." Trends in Cell Biology **16**(12): 649.

Lo, H. W., S. C. Hsu, et al. (2005). "Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway." Cancer Cell **7**(6): 575-89.

Lo, H. W., W. Xia, et al. (2005). "Novel prognostic value of nuclear epidermal growth factor receptor in breast cancer." Cancer Res **65**(1): 338-48.

Madeo, F., J. Schlauer, et al. (1998). "Tyrosine phosphorylation regulates cell cycle-dependent nuclear localization of Cdc48p." Mol Biol Cell **9**(1): 131-41.

Martin-Nieto, J. and A. Villalobo (1998). "The human epidermal growth factor receptor contains a juxtamembrane calmodulin-binding site." Biochemistry **37**(1): 227-36.

Mattoon, D., P. Klein, et al. (2004). "The tethered configuration of the EGF receptor extracellular domain exerts only a limited control of receptor function." PNAS **101**(4): 923-928.

McLaughlin, S., S. O. Smith, et al. (2005). "An electrostatic engine model for autoinhibition and activation of the epidermal growth factor receptor (EGFR/ErbB) family." J. Gen. Physiol. **126**: 41-53.

McLaughlin, S., S. O. Smith, et al. (2005). "An electrostatic engine model for autoinhibition and activation of the epidermal growth factor receptor (EGFR/ErbB) family." J Gen Physiol **126**(1): 41-53.

Mendrola, J. M., M. B. Berger, et al. (2002). "The Single Transmembrane Domains of ErbB Receptors Self-associate in Cell Membranes." J. Biol. Chem. **277**(7): 4704-4712.

Miettinen, P. J., J. E. Berger, et al. (1995). "Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor." Nature **376**(6538): 337.

Mol, C. D., K. B. Lim, et al. (2003). "Structure of a c-kit product complex reveals the basis for kinase transactivation." J. Biol. Chem. **278**: 31461-31464.

Mori-Konya, C., N. Kato, et al. (2009). "p97/valosin-containing protein (VCP) is highly modulated by phosphorylation and acetylation." Genes Cells **14**(4): 483-97.

Mulloy, R., A. Ferrand, et al. (2007). "Epidermal Growth Factor Receptor Mutants from Human Lung Cancers Exhibit Enhanced Catalytic Activity and Increased Sensitivity to Gefitinib." Cancer Res **67**(5): 2325-2330.

Ni, C. Y., M. P. Murphy, et al. (2001). "gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase." Science **294**(5549): 2179-81.

Ogiso, H., R. Ishitani, et al. (2002). "Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains." Cell **110**(6): 775-87.

Osborne, A. R., T. A. Rapoport, et al. (2005). "Protein translocation by the Sec61/SecY channel." Annu Rev Cell Dev Biol **21**: 529-50.

Pallis, A. G., A. Voutsina, et al. (2007). "'Classical' but not 'other' mutations of EGFR kinase domain are associated with clinical outcome in gefitinib-treated patients with non-small cell lung cancer." Br. J. Cancer **97**: 1560-1566.

Pallis, A. G., A. Voutsina, et al. (2007). "'Classical' but not 'other' mutations of EGFR kinase domain are associated with clinical outcome in gefitinib-treated patients with non-small cell lung cancer." Br J Cancer **97**(11): 1560-6.

Qiu, C., M. K. Tarrant, et al. (2008). "Mechanism of Activation and Inhibition of the HER4/ErbB4 Kinase." Structure **16**: 460-467.

Rappoport, J. Z. and S. M. Simon (2009). "Endocytic trafficking of activated EGFR is AP-2 dependent and occurs through preformed clathrin spots." J Cell Sci **122**(Pt 9): 1301-5.

Red Brewer, M., S. H. Choi, et al. (2009). "The juxtamembrane region of the EGF receptor functions as an activation domain." Mol Cell **34**(6): 641-51.

Riese, D. J., 2nd and D. F. Stern (1998). "Specificity within the EGF family/ErbB receptor family signaling network." Bioessays **20**(1): 41-8.

Ruddon, R. W. (2007). Cancer biology. Oxford ; New York, Oxford University Press.

Russo, M. W., T. J. Lukas, et al. (1985). "Identification of residues in the nucleotide binding site of the epidermal growth factor receptor/kinase." J Biol Chem **260**(9): 5205-8.

Sardi, S. P., J. Murtie, et al. (2006). "Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain." Cell **127**(1): 185-97.

Schneider, M. R. and E. Wolf (2009). "The epidermal growth factor receptor ligands at a glance." J Cell Physiol **218**(3): 460-6.

Segatto, O., C. R. King, et al. (1988). "Different structural alterations upregulate in vitro tyrosine kinase activity and transforming potency of the erbB-2 gene." Mol Cell Biol **8**(12): 5570-4.

Shah, N. P., J. M. Nicoll, et al. (2002). "Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia." Cancer Cell **2**(2): 117-25.

Sharma, S. V., D. W. Bell, et al. (2007). "Epidermal growth factor receptor mutations in lung cancer." Nat Rev Cancer **7**(3): 169-81.

Sharma, S. V., D. W. Bell, et al. (2007). "Epidermal growth factor receptor mutations in lung cancer." Nat. Rev. Cancer **7**(3): 169-181.

Sharpe, S., K. R. Barber, et al. (2002). "Interaction between ErbB-1 and ErbB-2 transmembrane domains in bilayer membranes." FEBS Lett **519**(1-3): 103-7.

Sibilia, M. and E. F. Wagner (1995). "Strain-dependent epithelial defects in mice lacking the EGF receptor." Science **269**(5221): 234-238.

Song Jae Kil, C. C. (2000). "EGF receptor residues Leu(679), Leu(680) mediate selective sorting of ligand-receptor complexes in early endosomal compartments." J. Cell. Physiol. **185**(47-60).

Song Jae Kil, C. C. (2000). "EGF receptor residues Leu(679), Leu(680) mediate selective sorting of ligand-receptor complexes in early endosomal compartments." Journal of Cellular Physiology **185**(1): 47-60.

Sorkin, A. and L. K. Goh (2009). "Endocytosis and intracellular trafficking of ErbBs." Exp Cell Res **315**(4): 683-96.

Sorkin, A. and M. Von Zastrow (2002). "Signal transduction and endocytosis: close encounters of many kinds." Nat Rev Mol Cell Biol **3**(8): 600-14.

Stacey, D. W. (2003). "Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells." Curr Opin Cell Biol **15**(2): 158-63.

Stamos, J., M. X. Sliwkowski, et al. (2002). "Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor." J. Biol. Chem. **277**: 46265-46272.

Stang, E., F. D. Blystad, et al. (2004). "Cbl-dependent ubiquitination is required for progression of EGF receptors into clathrin-coated pits." Mol Biol Cell **15**(8): 3591-604.

Summerfield, A. E., A. K. Hudnall, et al. (1996). "Identification of residues of the epidermal growth factor receptor proximal to residue 45 of bound epidermal growth factor." J Biol Chem **271**(33): 19656-9.

Takishima, K., I. Griswold-Prenner, et al. (1991). "Epidermal growth factor (EGF) receptor T669 peptide kinase from 3T3-L1 cells is an EGF-stimulated "MAP" kinase." PNAS **88**(6): 2520-2524.

Thiel, K. W. and G. Carpenter (2007). "Epidermal growth factor receptor juxtamembrane region regulates allosteric tyrosine kinase activation." Proc Natl Acad Sci U S A **104**(49): 19238-43.

Thiel, K. W. and G. Carpenter (2007). "Epidermal growth factor receptor juxtamembrane region regulates allosteric tyrosine kinase activation." Proc. Natl. Acad. Sci. U. S. A. **104**: 19238-19243.

Threadgill, D. W., A. A. Dlugosz, et al. (1995). "Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype." Science **269**(5221): 230-4.

Trombetta, E. S. and A. J. Parodi (2003). "Quality control and protein folding in the secretory pathway." Annu Rev Cell Dev Biol **19**: 649-76.

Tsao, M. S., A. Sakurada, et al. (2005). "Erlotinib in lung cancer - molecular and clinical predictors of outcome." N. Engl. J. Med. **353**: 133-144.

Ullrich, A., L. Coussens, et al. (1984). "Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells." Nature **309**(5967): 418-25.

Vandermoere, F., I. El Yazidi-Belkoura, et al. (2006). "The valosin-containing protein (VCP) is a target of Akt signaling required for cell survival." J Biol Chem **281**(20): 14307-13.

Vieira, A. V., C. Lamaze, et al. (1996). "Control of EGF receptor signaling by clathrin-mediated endocytosis." Science **274**(5295): 2086-9.

Walton, G. M., W. S. Chen, et al. (1990). "Analysis of deletions of the carboxyl terminus of the epidermal growth factor receptor reveals self-phosphorylation at tyrosine 992 and enhanced in vivo tyrosine phosphorylation of cell substrates." J. Biol. Chem. **265**(3): 1750-1754.

Wang, Q., L. Li, et al. (2008). "Inhibition of p97-dependent protein degradation by Eeyarestatin I." J Biol Chem **283**(12): 7445-54.

Wang, Q., C. Song, et al. (2004). "Molecular perspectives on p97-VCP: progress in understanding its structure and diverse biological functions." J Struct Biol **146**(1-2): 44-57.

Welsh, J. B., G. N. Gill, et al. (1991). "A negative feedback loop attenuates EGF-induced morphological changes." J. Cell Biol. **114**(3): 533-543.

Wilbrey, A. L., J. E. Haley, et al. (2008). "VCP binding influences intracellular distribution of the slow Wallerian degeneration protein, Wld(S)." Mol Cell Neurosci **38**(3): 325-40.

Woltjer, R. L., T. J. Lukas, et al. (1992). "Direct identification of residues of the epidermal growth factor receptor in close proximity to the amino terminus of bound epidermal growth factor." Proc Natl Acad Sci U S A **89**(16): 7801-5.

Wong, A. J., J. M. Ruppert, et al. (1992). "Structural alterations of the epidermal growth factor receptor gene in human gliomas." Proc Natl Acad Sci U S A **89**(7): 2965-9.

Wood, E. R., L. M. Shewchuk, et al. (2008). "6-ethynylthieno[3,2-d]- and 6-ethynylthieno[2,3-d]pyrimidin-4-anilines as tunable covalent modifiers of ErbB kinases." Proc. Natl. Acad. Sci. U. S. A. **105**: 2773–2778.

Wood, E. R., A. T. Truesdale, et al. (2004). "A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells." Cancer Res. **64**: 6652-6659.

Wybenga-Groot, L. E., B. Baskin, et al. (2001). "Structural basis for autoinhibition of the Ephb2 receptor tyrosine kinase by the unphosphorylated juxtamembrane region." Cell **106**: 745-757.

Yamazaki, H., Y. Fukui, et al. (1988). "Amplification of the structurally and functionally altered epidermal growth factor receptor gene (c-erbB) in human brain tumors." Mol Cell Biol **8**(4): 1816-20.

Yarden, Y. and M. X. Sliwkowski (2001). "Untangling the ErbB signalling network." Nat Rev Mol Cell Biol **2**(2): 127-37.

Ye, Y. (2006). "Diverse functions with a common regulator: ubiquitin takes command of an AAA ATPase." J Struct Biol **156**(1): 29-40.

Ye, Y., H. H. Meyer, et al. (2001). "The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol." Nature **414**(6864): 652-6.

Ye, Y., Y. Shibata, et al. (2004). "A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol." Nature **429**(6994): 841-7.

Yun, C.-H., T. J. Boggon, et al. (2007). "Structures of Lung Cancer-Derived EGFR Mutants and Inhibitor Complexes: Mechanism of Activation and Insights into Differential Inhibitor Sensitivity." Cancer Cell **11**(3): 217.

Yun, C. H., T. J. Boggon, et al. (2007). "Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity." Cancer Cell **11**(3): 217-27.

Yun, C. H., T. J. Boggon, et al. (2007). "Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity." Cancer Cell **11**: 217-227.

Zawrocki, A. and W. Biernat (2005). "Epidermal growth factor receptor in glioblastoma." Folia Neuropathol **43**(3): 123-32.

Zhang, X., J. Gureasko, et al. (2006). "An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor." Cell **125**(6): 1137-49.

Zhang, X., K. A. Pickin, et al. (2007). "Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface." Nature **450**(7170): 741-4.

Zhao, G., X. Zhou, et al. (2007). "Studies on peptide:N-glycanase-p97 interaction suggest that p97 phosphorylation modulates endoplasmic reticulum-associated degradation." Proc Natl Acad Sci U S A **104**(21): 8785-90.