Beyond the Canonical Function of Receptor Tyrosine Kinase: New Perspectives for ERBB2 (HER2) Signaling Control Across the Cell Membrane

Qian Zhang
*University of Miami, qzhang@med.miami.edu*

Follow this and additional works at: [https://scholarlyrepository.miami.edu/oa_dissertations](https://scholarlyrepository.miami.edu/oa_dissertations)

**Recommended Citation**
Zhang, Qian, 'Beyond the Canonical Function of Receptor Tyrosine Kinase: New Perspectives for ERBB2 (HER2) Signaling Control Across the Cell Membrane' (2014). Open Access Dissertations. 1206.
[https://scholarlyrepository.miami.edu/oa_dissertations/1206](https://scholarlyrepository.miami.edu/oa_dissertations/1206)
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

BEYOND THE CANONICAL FUNCTION OF RECEPTOR TYROSINE KINASE:
NEW PERSPECTIVES FOR ERBB2 (HER2) SIGNALING CONTROL ACROSS
THE CELL MEMBRANE

Qian Zhang

Approved:

Ralf Landgraf, Ph.D.
Associate Professor of Biochemistry and Molecular Biology

Xiangxi Xu, Ph.D
Professor of Cell Biology

Feng Gong, Ph.D.
Associate Professor of Biochemistry and Molecular Biology

M. Brian Blake, Ph.D.
Dean of the Graduate School

Thomas K. Harris, Ph.D.
Associate Professor of Biochemistry and Molecular Biology
The complex of ligand-binding deficient ERBB2 with kinase-dead ERBB3 represents the most potent signaling partner among the ERBB receptor family. The canonical model of receptor activation only provides an explanation for ERBB3 phosphorylation, leaving the ERBB2 part unresolved. Traditional therapeutic agents including monoclonal antibodies or kinase inhibitors against ERBB2 are effective in treatment, but their mechanism of action remains unknown. Meanwhile, patients often develop high levels of drug resistance over time. Since the process of ERBB2 signaling activation and transduction is elusive, we are interested in looking into the underlying molecular mechanisms, and thereby developing new medications in an effort to treat ERBB2-overexpressed breast cancer.

Higher order complexes of ERBB receptors have been observed biophysically and offer a theoretical route for ERBB2 phosphorylation, but it is not clear whether such complexes provide functionality beyond the constituent dimers. In my first project, we show that a previously selected inhibitory RNA aptamer which targets the extracellular domain (ECD) of ERBB3 acts by sterically disrupting
these higher order interactions. Ligand binding, heterodimerization, phosphorylation of ERBB3, and AKT signaling are only minimally affected, whereas ERBB2 phosphorylation and MAPK signaling are selectively inhibited. The mapping of the binding sites and the creation of aptamer-resistant point mutants are consistent with a model of side-by-side oriented heterodimers to facilitate proxy phosphorylation. Additional modes of signaling with relevance to pathological ERBB expression states emerge at high receptor levels. Hence, higher order complexes of nonoverexpressed ERBB receptors are an integral and qualitatively distinct part of normal ERBB2/ERBB3 signaling.

Earlier literature has indicated Serine/Threonine phosphorylation at the carboxyl terminus of EGFR could promote receptor desensitization and reduce tyrosine kinase activity, which associates with maintaining the efficacy of chemotherapy-induced cell death. However, the details of serine/threonine phosphorylation profile at ERBB2 C-tail remain unknown. In the second project, I identified multiple serine/threonine phosphorylation sites by mass spectrometry at ERBB2 carboxyl end and evaluated their role in inhibition of receptor function. Our preliminary results suggest the possibility of p38 as a candidate responsible for phosphorylating these serine residues. Since phospho-serine is capable of retaining ERBB2 at relatively low activation status, either by changing receptor allosteric activation, or increasing its internalization, it is feasible to design drugs aimed at inducing the serine phosphorylation signaling.
Besides receptor activation control from its phosphorylated status, the receptor can also interact with intracellular proteins under quiescent or unphosphorylated state. Grb7 is coamplified with ERBB2 on chromosome 17q12 in ERBB2 overexpression breast cancer. It can exist in equilibrium between monomer and dimer and the dimer can be disrupted by single point mutation. It is possible that the shift of this equilibrium can result in differences in adaptor recruitment to receptors. We ask the question how Grb7 is recruited to ERBB2/3 complex mechanistically and how the potential differences in recruitment impact the tumorigenesis of ERBB2. Our CoIP studies identified a novel second mode of Grb7 interaction with ERBB2, but not with ERBB3. Combined with the receptor higher structure activation model and the dimerization properties of Grb7, we predict the dual binding mode may add to the signaling diversity of ERBB2/ERBB3 complex.
# Table of Contents

List of Figures .................................................................................................................. vi  
List of Tables .................................................................................................................... viii  

Chapter  
1. **Introduction** .............................................................................................................. 1  
   1.1 Breast cancer epidemiology .................................................................................. 1  
   1.2 ERBB family receptors related breast cancer development ............................... 2  
      1.2.1 ERBB receptor structure .............................................................................. 3  
      1.2.2 ERBB ligands ............................................................................................. 3  
      1.2.3 ERBB signaling pathways .......................................................................... 5  
      1.2.4 Receptor cross-talk and signaling attenuation ............................................ 7  
   1.3 Canonical views for receptor activation control ............................................... 9  
      1.3.1 The ligand induced dimerization model ..................................................... 9  
      1.3.2 Dimerization based receptor activation model .......................................... 10  
      1.3.3 Clinical drugs to treat ERBB associated cancer ....................................... 11  
   1.4 Beyond the canonical function of ERBB receptors .......................................... 14  
      1.4.1 Receptor higher order structure .................................................................. 14  
      1.4.2 Receptor activation control from regions other than ECD and kinase domain ........................................................................................................... 16  
      1.4.3 Adaptors/effector proteins binding to the quiescent/unphosphorylated state of receptors ......................................................................................... 17  
   1.5 The focus of my study and three open questions .............................................. 18  
2. **Functional Isolation of Activated and Unilaterally Phosphorylated Heterodimers of ERBB2 and ERBB3 as Scaffolds in Ligand-Dependent Signaling** ................................................................................................................................. 25  
   2.1 Summary .............................................................................................................. 25  
   2.2 Background ........................................................................................................ 26  
   2.3 Materials and methods ..................................................................................... 29  
   2.4 Results ................................................................................................................ 31  
      2.4.1 The ERBB3-directed aptamer selectively inhibits ligand-induced phosphorylation of ERBB2 but not the formation of active heterodimers ........ 31
2.4.2 Where does A30 bind relative to known interacting surfaces of ERBB receptors? .......................................................................................................................................................... 34
2.4.3 Overexpression shifts the dynamics of proxy phosphorylation .............................................................. 38
2.5 Conclusion and discussion .................................................................................................................................................................................. 40

3. p38 Mediated Serine Phosphorylation of ERBB2 at Receptor Carboxyl tail is Inhibitory to ERBB2 Tyrosine Phosphorylation .......................................................... 51
3.1 Summary ............................................................................................................................................................................................ 51
3.2 Background ........................................................................................................................................................................................ 52
3.3 Materials and methods ........................................................................................................................................................................... 54
3.4 Results ........................................................................................................................................................................................................ 56
3.4.1 Multiple serine/threonine phosphorylation sites were identified at ERBB2 carboxyl terminal tail and they were associated with receptor tyrosine phosphorylation level .................................................................................................................................................................................. 56
3.4.2 p38 might be the kinase responsible for phosphorylating multiple serine residues at ERBB2 carboxyl tail ................................................................................................................................................................................................. 60
3.5 Discussion ........................................................................................................................................................................................... 61

4. GRB7 is recruited to ERBB2 through two distinct modes: phosphotyrosine dependent binding mediated by GRB7-SH2 domain and phosphotyrosine independent binding probably mediated by GRB7-BPS region .............. 65
4.1 Summary ............................................................................................................................................................................................ 65
4.2 Background ........................................................................................................................................................................................ 66
4.3 Materials and methods ........................................................................................................................................................................... 68
4.4 Results ........................................................................................................................................................................................................ 70
4.4.1 GRB7 can bind to non-phosphorylated ERBB2 through a novel mechanism independent on SH2 domain .................................................................................................................................................................................. 70
4.4.2 Focal Adhesion Kinase (FAK) may play a role in the ERBB2 signaling pathway leading to GRB7 phosphorylation .................................................................................................................................................................................. 72
4.4.3 GRB7 is recruited to ERBB3 only in phosphotyrosine-dependent manner, and this interaction is increased upon ligand stimulation ................................................................................................................................................................................................. 74
4.5 Discussion ........................................................................................................................................................................................ 75
5. Discussion and Significance ................................................................. 78
6. References ............................................................................................. 88
List of Figures

Figure 1.1 Female breast anatomy ................................................................. 2
Figure 1.2 Structural presentation of ERBB receptors ............................... 4
Figure 1.3 ERBB signaling pathways .......................................................... 6
Figure 1.4 Conformation of the extracellular domains of ERBB receptors .... 9
Figure 1.5 EGFR allosteric activation ............................................................. 11
Figure 1.6 The mechanism of inhibition by Trastuzumab and Pertuzumab .... 13
Figure 1.7 Higher order receptor association and activation model for EGFR and ERBB2/ERBB3 ............................................................... 15
Figure 1.8 Proposed models for ERBB2 phosphorylation ......................... 20
Figure 1.9 GRB7 recruitment leads to both cell proliferation and cell migration.. 22
Figure 2.1 A30 preferentially inhibits ERBB2 phosphorylation but not heterodimerization with ERBB3 ............................................................. 33
Figure 2.2 The preferential inhibition of ERBB2 correlates with a pronounced inhibition of MAPK activation, whereas both ERBB3 phosphorylation and AKT activation are largely resistant to A30 ...................................................... 34
Figure 2.3 A30 targets the domain III–IV junction region ......................... 37
Figure 2.4 Over expression shifts the dynamics of proxy phosphorylation ..... 38
Figure 2.5 Model for receptor phosphorylation in higher order complex ........ 43
Figure S2.1 Efficient inhibition of ERBB2 phosphorylation (Tyr1139) by A30 relies on the steric interference by its nonbinding tail region ......................... 47
Figure S2.2 A30 targets the junction of domains III and IV in the ERBB3 ECD but not the dimerization interface, and A30 is not in competition with NRG .... 48
Figure S2.3 A30 binding and its loss in ERBB3 mutants is independent of receptor density ................................................................. 49
Figure S2.4 Inhibition of constitutive and ligand-induced receptor phosphorylation in SKBr breast cancer cells ......................................................... 49
Figure S2.5 The observed inhibition exerted by pertuzumab (2C4) is not limited by antibody concentration ......................................................... 49
Figure S2.6 Phosphorylation of ERBB2 at Tyr1139 is correlated but not critical for efficient MAPK activation .......................................................... 50
Figure 3.1 The identification of phospho-serine residues at ERBB2 carboxyl terminal

Figure 3.2 Serine to Alanine mutation at the carboxyl tail of ERBB2 elevates ERBB2 tyrosine phosphorylation

Figure 3.3 Single mutation of Ser->Ala at C-terminal tail of ERBB2 increases pERBB2 under IP condition

Figure 3.4 Testing of p38 as a potential kinase to phosphorylate serine residues at ERBB2 carboxyl tail

Figure 3.5 Proposed mechanism for p38-mediated inhibition of ERBB2 tyrosine phosphorylation through serine phosphorylation

Figure 4.1 Domain components of GRB7 family adaptors

Figure 4.2 FAK-GRB7-cell migration signaling

Figure 4.3 GRB7 binds to ERBB2 in a novel receptor phosphotyrosine independent manner

Figure 4.4 Phosphotyrosine level of GRB7 mediated by ERBB2 signaling is inhibited by FAK inhibitor

Figure 4.5 Only phosphorylated ERBB3 is capable of recruiting GRB7

Figure 5.1 A proposed model for dimerized GRB7 to associate with receptor higher order complex through multi-domain binding modes

Figure 5.2 A proposed model of ERBB2/ERBB3 signaling mediated GRB7 tyrosine phosphorylation by FAK
List of Table

Table 1 Serine/Threonine phosphorylation sites identified at EGFR and ERBB2 carboxyl terminal tail. ..........................................................................................................................54
Chapter 1 Introduction

1.1 Breast cancer epidemiology

Breast cancer is a malignant condition of the breast cells that may invade into adjacent tissues or propagate to other organs of the body. It includes carcinoma that begins in epithelial cells and sarcoma that arises from fat/muscle tissues or blood vessels. The female breast consists of lobules which produces milk, ducts which delivers milk to the nipple, and stroma tissues (Figure 1.1). In most cases, breast cancer occurs within ductal cells, but some can also start in lobular cells (Pike, Spicer et al. 1993). Breast cancer is the most frequently happening and the second leading cause of cancer death among women in the United States. 12% of American women will be diagnosed with breast cancer through their lifetime. In 2013, it is expected that 232,340 new cases of invasive breast cancer and 64,640 in situ cancer patients will be identified among US females (DeSantis, Ma et al. 2014). A variety of risk factors like gender, family history, early menarche/late menopause, age, genetic mutations, life style, obesity and hormone consumption are related to breast cancer occurrence (McPherson, Steel et al. 2000). According to cancer statistics, about 20%-30% of breast cancers have overexpressed ERBB2 which induces cell growth and proliferation. This type of cancer can develop fast and highly metastasize to other sites, so they are correlated with very poor long-term prognosis (Kallioniemi, Holli et al. 1991). More importantly, since the ligand-unbound ERBB2 and kinase-deficient ERBB3 represent the most mitogenic signaling pair among ERBB family
receptors, we focused most of our effort on investigating ERBB2 related breast cancer signaling across the cell membrane.

Figure 1.1 Female breast anatomy.
(http://www.cancer.gov/cancertopics/pdq/prevention/breast/Patient/page2)

1.2 ERBB family receptors related breast cancer development

ERBB receptor family, including ERBB1(EGFR), ERBB2 (HER2), ERBB3 (HER3), ERBB4 (HER4) is a group of structurally related type I receptor tyrosine kinase. The signaling of these proteins is implicated with cell growth, neural maintenance, and organ development. Excessive ERBB signaling will induce tumorigenesis. In contrast to ERBB3 and ERBB4, overexpression of EGFR and ERBB2 is associated with various types of invasive cancers in human (Verbeek, Adriaansen-Slot et al. 1998). Downregulated ERBB signaling is frequently correlated with deficient development of human organs, such as heart, brain, lung and even skin (Burgess 2008). Despite of the important role ERBB receptors play in maintaining the normal function of human body, their molecular
structure and signaling network are poorly understood, especially compared to the abundance of data for cancer scenarios.

1.2.1 ERBB receptor structure

All ERBB receptors have a conserved structure consisting of the extracellular domain (ECD), transmembrane span, a junxtamembrane region, kinase domain (involving N and C lobes) and carboxyl terminal tail (Figure 1.2 A). The ligand-interacting ECD has four subdomains known as L1 (leucine-rich repeats 1), CR1 (cysteine-rich 1), L2 and CR2 (or in simple terms I – IV). Domains I and III are responsible for ligand binding and the dimerization loop within domain II is critical for receptor – receptor association (Figure 1.2 B) (Ferguson 2008). Both EGFR and ERBB4 have potent kinase activity and intact ligand binding ability, so they constitute fully functional receptors. ERBB3 lacks kinase activity due to a point mutation on key residues in the catalytic region of the kinase domain. ERBB2, on the other hand, has potent kinase activity but is deficient in liganding binding. Hence, ERBB2 is normally paired with other receptor members to activate signaling and ERBB2/ERBB3 represents the most potent mitogenic complex.

1.2.2 ERBB ligands

The ligands for ERBB receptors are small protein growth factors that bind to the ERBB receptors and stimulate signaling activation. On cell surface, mature ligands are produced from precursors through proteolytic cleavage by ADAM family of metalloproteases (Blobel 2005). All ligands contain an EGF-like core domain of 60 amino acids which is sufficient for receptor binding and basic activation (Jones, Akita et al. 1999). Depending on their binding specificity to
ERBBs, ligands are divided into an EGF related group that binds to EGFR and neuregulins (NRGs) that interacts with ERBB3 and ERBB4 (Warren and Landgraf 2006). EGFR is recognized by EGF, transforming growth factor α (TGF-α), amphiregulin (AR), β-cellulin (BTC), epiregulin (EPR), and heparin binding EGF-like growth factor (HB-EGF). ERBB3 binds neuregulin-1. ERBB4 agonists include epiregulin, HB-EGF and neuregulins (Figure 1.3) (Yarden and Sliwkowski 2001). Neuregulins are derived from four genes (NRG 1-4), each of which exists in multiple splicing isoforms. The ligands diversity and recognition specificity can add to the complexity of ERBB signaling outcomes.

Figure 1.2. Structural presentation of ERBB receptors. A. The structure of EGFR receptor comprises four extracellular domains I-IV (L1, CR1, L2, CR2), transmembrane span, juxtamembrane region, kinase domain, and C-terminal tail. B. Ribbon diagrams of EGFR domains. Domains I and III adopt a β-helix fold. Domains II and IV adopt extended structures comprising a series of disulfide-bonded modules. The inactive kinase is shown with the ATP analogue (AMP-PNP) in stick representation. (Kathryn M. Ferguson 2008)
1.2.3 ERBB signaling pathways

ERBB receptors are able to recruit multiplex downstream adaptor molecules or catalytic enzymes to further signaling transduction cascades (Figure 1.3). The putative interaction mode is the binding of SH2 domain containing proteins to phosphotyrosine docking sites at receptor carboxyl terminal tail (Schlessinger 1994). The GRB2-SOS-Ras pathway can integrate incoming signaling from all combinations of active receptor pairs, while PI3K-Akt cascade is mainly coupled to ERBB3 containing hetero-complex (Rubin and Yarden 2001, Gschwind, Fischer et al. 2004). PLCγ-PKC mediated stress activated protein kinase cascade and cell migration related Vav-Rac-JNK signaling are two other major pathways involved in the signaling-processing layer (Rubin and Yarden 2001).

Compared to the EGFR homodimer which has the broadest range of downstream binding proteins, the kinase-deficient ERBB3 has low complexity but high potency in terms of interaction with adaptors mediated through tandem repeats. Following activation of multiplex signaling cascades, gene transcription programs will be regulated by transcription factors in the nucleus. The proto-oncogenes (jun, myc, fos), zinc-finger-containing transcriptional factors (Egr1, Sp1), and Ets family members (GABP) are among the key regulators (Schaeffer, Duclert et al. 1998).
All together, the recognition specificity of ligands, various engagements of the adaptor proteins, structural characteristics of receptors as well as the phosphotyrosine profile at the tail region, gene transcription control by distinct effectors, and the different duration of each signaling pathway can synthetically determine the outcomes of ERBB signaling. Cellular responses to ERBB signaling vary from growth, proliferation and differentiation, to not only adhesion, migration and tumorigenesis, but also to apoptosis, cell death, and anoikis resistance.
1.2.4 Receptor cross-talk and signaling attenuation

The cross-talk of ERBB with other signaling pathways also adds to the diversity of signaling network. Those ERBB independent signals include neurotransmitters, hormones, cytokines and stress responsive inducers, most of which directly interact with ERBB receptors and modify their kinase activity or endocytic properties (Schaeffer, Duclert et al. 1998). For example, activation of G protein-coupled receptors (GPCR) by agonists such as lysophosphatidic acid (LPA), thrombin, and angiotensin-II can trigger EGFR activation through stimulation of ADAM family metalloproteinases in an effort to increase local EGF concentration (Prenzel, Zwick et al. 1999, Carpenter 2000, Gschwind, Zwick et al. 2001, Gschwind, Prenzel et al. 2002). The similar mechanism was utilized by Frizzled ligands Wnt1 and Wnt5a to modulate EGFR activation (Civenni, Holbro et al. 2003). c-Src can be activated by stimulated GPCRs, resulting in its direct phosphorylation of residues at EGFR C-terminal tail (Biscardi, Maa et al. 1999).

In addition, a positive feedback loop exists between integrin α6β4 and ERBB2. ERBB2 induces integrin α6β4 phosphorylation in c-Src mediated manner, leading to triple complex formation of ERBB2-Src-integrin α6β4, which finally promotes further activation of ERBB2 (Chung and Kim 2008).

Since deregulated signaling causes aberrant cell proliferation that will lead to human diseases, mammalian cells have developed complex regulatory mechanisms to turn off the signaling. This negative regulation process is basically approached by three ways: dephosphorylation of tyrosine residues, internalization and degradation of activated receptors, and modulation of receptor
kinase activity. Phosphatases, such as density-enhanced phosphatase-1 (DEP-1) and protein tyrosine phosphatase 1B (PTP1B), can dephosphorylate the phosphorylated tyrosine residues at receptor tail region, such that adaptor protein recruitment to the cell surface receptors is diminished (Tarcic, Boguslavsky et al. 2009, Yip, Saha et al. 2010). For receptor internalization, two consequences of receptor degradation and recycling are identified. In its resting state, EGFR mainly resides in caveolae on cell membrane. Upon ligand binding, it exits caveolae and enters clathrin-coated pits, which undergoes endocytosis through endosomes. Unliganded EGFR is sorted by endosomes and sent back to cell surface, whereas ligand occupied receptor is tagged by the E3 ubiquitin ligase Cbl and directed to degradation in lysosomes (Levkowitz, Waterman et al. 1998). By modulating receptor kinase activity, oncogenic signaling can also be switched off. An example of this is shown by the interaction of receptor kinase with heat shock protein Hsp90. Hsp90 binding to ERBB2 blocks phosphorylation of ERBB2 at residue Tyr877 within the receptor kinase domain (Xu, Yuan et al. 2007). Its binding to ERBB2 stabilizes the receptor which has been proposed as an explanation why high Hsp90 levels correlate with elevated ERBB2 and a poor prognosis. Likewise, ERBB2 overexpressing cancers are highly sensitive to Hsp90 inhibition. (Pick, Kluger et al. 2007).
1.3 Canonical views for receptor activation control

1.3.1 The ligand induced dimerization model

The crystal structure study of extracellular domains of all four ERBB members revealed a similar conformation for EGFR, ERBB3, and ERBB4, but a distinct one for ERBB2 (Cho and Leahy 2002, Ogiso, Ishitani et al. 2002, Burgess, Cho et al. 2003, Ferguson, Berger et al. 2003, Bouyain, Longo et al. 2005). For EGFR, a tethered and autoinhibited conformation of ECD is in a dynamic equilibrium with an extended form. EGF binds preferentially to the extended forms of the receptor (where domains I and III can both contact the ligand), shifting the equilibrium toward dimerization, resulting in autophosphorylation and stimulation of protein tyrosine kinase activity (Mattoon, Klein et al. 2004). As shown in figure 1.4, for the tethered state, subdomain II and IV are interacting to establish a conformation that is incompatible with dimerization. However, ERBB2 is an exception in that its extracellular domain is constitutively extended and

Figure 1.4 Conformation of the extracellular domains of ERBB receptors.
dimerization competent (Garrett, McKern et al. 2003). In the canonical ligand-induced dimerization model, the receptors form homo- or heterodimers upon ligand stimulation (Ferguson 2008). Since ERBB2 does not bind to ligand and barely homodimerizes, it usually interacts with its binding partner to promote signaling.

1.3.2 Dimerization based receptor activation model

As an additional level of control, the intracellular region of ERBB receptor also exists in an autoinhibited state. The cocrystal structure of EGFR kinase domain with AMP-PNP shows an inactive conformation which is identical to the therapeutic drug lapatinib bound receptor. This conformation resembles that of (cyclin-dependent kinases)CDK/Src family kinases (Bose and Zhang 2009). Helix αC in the kinase domain is rotated outwardly with respect to the rest of the kinase domain, such that the N-terminal portion of the activation loop is packed against the helix to stabilize it. This packing is mediated by two leucine residues (L834 and L837) in the activation loop which interacts with hydrophobic sites on helix αC. These interactions stabilize the CDK/Src-like conformation by sterically blocking the formation of the Lys721–Glu738 salt bridge which is fundamental to catalytic activity (Huse and Kuriyan 2002, Zhang, Gureasko et al. 2006). EGFR with Leu834 and Leu837 mutated to arginine and glutamine is frequently associated with drug resistant non-small cell lung cancer (Zhang, Gureasko et al. 2006).

In the active conformation of the EGFR kinase domain, an asymmetric dimer forms in which the C-lobe of one receptor kinase domain (activator) makes
contacts with the N-lobe of another receptor kinase domain (receiver). The activator induces conformational change in the N-lobe of the receiver such that the displacement of helix αC in the receiver kinase will disrupt its salt bridge interaction with the activation loop. The release of the activation loop from helix αC will make space for the coordination of an ATP substrate into the catalytic site. All members of the ERBB family receptors can serve as activator to stimulate its dimerization partner, whereas only EGFR, ERBB2 and ERBB4 can act as receiver. ERBB3 has point mutations at key residues within its kinase domain, resulting in abolished kinase activity (Jura, Shan et al. 2009). Moreover, since it has divergent sequence at the N-lobe compared to other receptors, it can only activate other members but not the other way round (Figure 1.5).

![Figure 1.5 EGFR allosteric activation](image)

Figure 1.5 EGFR allosteric activation. The kinase domain is intrinsically autoinhibited. The EGFR forms an asymmetric dimer upon ligand binding in which the C-lobe of one kinase domain, called the “Activator,” makes contacts with the N-lobe of the second kinase domain, called the “Receiver.” These contacts induce conformational changes in the N-lobe of the Receiver kinase to disrupt autoinhibitory interactions. As a result, the Receiver kinase can adopt the active configuration to phosphorylate the activator.

1.3.3 Clinical drugs to treat ERBB associated cancer

Given the well-studied mechanism of ERBB signaling and the critical role they play in tumor progression, the ERBB receptors are attractive drug targets from
therapeutic perspectives, especially EGFR and ERBB2 which are frequently overexpressed in multiple cancers.

**Antibodies**

Four antibodies targeting different regions in the receptor extracellular domains have been extensively studied and approved for clinical use. Cetuximab and related antibodies interact with domain III of EGFR so as to block binding of ligands to EGFR (Li, Schmitz et al. 2005, Li, Kussie et al. 2008). Matuzumab is another EGFR antagonist which also binds to domain III, but from a different direction and does not occlude ligand binding site on receptor. Structural studies of matuzumab Fab show that the antibody is oriented by to prevent the receptor from adopting the extended conformation needed for receptor dimerization. It blocks the positioning displacement of domain II relative to domain III that is known to be critical for removing the autoinhibition of extracellular regions (Schmiedel, Blaukat et al. 2008). The prevailing model of ligand-induced receptor dimerization implies that ERBB signaling could be blocked by an inhibitor capable of disrupting dimer formation. Pertuzumab is such an inhibitor binding to the dimerization loop of ERBB2. It can efficiently block ligand-stimulated heterodimerization of ERBB2 with both EGFR and ERBB3. However, pertuzumab does not interfere with ligand-independent ERBB2 tyrosine phosphorylation and more importantly, the tyrosine phosphorylation of ERBB3 in ERBB2 positive cells (Franklin, Carey et al. 2004). Trastuzumab is a potent drug in preventing cell proliferation of ERBB2 amplified breast cancer. It binds to domain IV of ECD and does not hinder ligand-induced ERBB2 heterodimerization.
Yet, in contrast to pertuzumab, trastuzumab is much more efficient in disrupting the ligand-independent phosphorylation of ERBB3 in ERBB2 overexpressed cells. In fact, the efficacy is directly linked to the inhibition of constitutive ERBB3 phosphorylation and AKT signaling (Junttila, Akita et al. 2009).

Figure 1.6 The mechanism of inhibition by Trastuzumab and Pertuzumab.
A. In ERBB2 overexpressed cells, ERBB2 interacts with ERBB3 independent of ligand. This leads to ERBB3 phophorylation and downstream activation of PI3K pathway. Trastuzumab inhibits the ligand-independent ERBB2/ERBB3 interaction, and ERBB3 phosphorylation. B. Pertuzumab can disrupt the ligand-induced association between ERBB2 and ERBB3 in both ERBB2 overexpressed and nonamplified cells. (Junttila, Akita et al. 2009)

Small molecule inhibitors
Another group of receptor tyrosine kinase (RTK) inhibitors is developed to inhibit the enzymatic activity of ERBB receptors. Gefitinib, erlotinib, and lapatinib are most commonly used clinic tyrosine kinase inhibitors (TKIs). While gefitinib and erlotinib bind to the active conformation of receptor kinase domain and compete directly with ATP, lapatinib binds to and freezes the inactive state (Moy and Goss 2006, Costa, Nguyen et al. 2008). Targeting other components of the ERBB signaling cascade including Src and Akt holds promise and has been tied with varying success (Brugge 1993).

1.4 Beyond the canonical function of ERBB receptors

Although the canonical ligand-induced dimerization model provides a basic paradigm for receptor activation control, results from recent studies lead to the modification of this model. The divergent signaling outcomes can not be reconciled based on simple dimer-driven receptor activation. Moreover, most of current RTK studies used EGFR as a model, but the underlying mechanism of EGFR signaling control in some cases does not apply to other ERBB receptors. In our study, we expanded the canonical model from the following three aspects.

1.4.1 Receptor higher order structure

One limitation of the canonical dimer based activation model is that it fails to explain the efficient phosphorylation of the ligand binding incompetent ERBB2 in complex with the kinase deficient ERBB3. It has been proposed that ERBB2 may be phosphorylated by other ERBB2 receptors within a larger complex (HUANG, OUYANG et al. 1998). Later study by Peter Nagy’s group provides evidence for the existence of receptor higher order structures. Flow-
A. In the absence of ligand, all receptors are in inactive states. Tethered (A) or extended (B) monomers are in equilibrium with tethered (C) or extended (D) dimers. Higher order oligomers (E and F) may also exist, although they are undetectable. Upon ligand binding the equilibrium shifts toward the tetrameric conformation of the receptor (G and H), which induces receptor activation.

B. ERBB2 forms large, inactive, and separate homoclusters in unstimulated cells, whereas ERBB3 form oligomers. Heregulin stimulation removes ERBB3 molecules from ERBB3 oligomers. Ligand-activated ERBB3 recruits ERBB2 proteins from inactive ERBB2 homoclusters to ERBB2-ERBB3 heterodimers.

cytometric homo-FRET measurements demonstrate that ERBB2 forms inactive homoclusters in unstimulated cells which dissociate upon stimulation. Combined with the fact that heregulin stimulation disrupts ERBB3 oligomers (Kani, Warren et al. 2005), this proposed model indicates that ligand-activated ERBB3 recruits ERBB2 proteins from inactive ERBB2 homoclusters, leading to the formation of high order structure of mixed ERBB2 and ERBB3 complexes with bound NRG in which both receptors become phosphorylated (Figure 1.7B) (Szabó, Horváth et al. 2008). In absence of ligand, EGFR is thought to exist in an equilibrium between tethered (A) or untethered (B) monomers and tethered (C) or untethered (D)
dimmers (Figure 1.7A). Higher order oligomers (E and F) may also be present. However, none of these ligand free receptors have significant tyrosine kinase activity. Upon ligand binding the equilibrium shifts toward the multimeric forms of the receptor (G and H) in which the inhibitory blocks are removed and the kinase activity is stimulated to induce receptor tyrosine phosphorylation (Clayton, Walker et al. 2005, Clayton, Orchard et al. 2008). Hence, dimerization is not the endpoint of receptor association and activation. Instead, higher order structures on cell membrane may pre-exist, create the context for efficient receptor phosphorylation to occur, and provide means to modulate the outcomes.

1.4.2 Receptor activation control from regions other than ECD and kinase domain

All of the transmembrane span, juxtamembrane linker, and cytoplasmic portions are implicated in impacting receptor activation. The presence of two GXXXG motifs in transmembrane region is related to receptor self-association and activation (Mendrola, Berger et al. 2002). The juxtamembrane region contributes to receptor activation by being directly involved in stabilizing the dimerization of EGFR kinase domain (Red Brewer, Choi et al. 2009). Since the carboxyl-terminal tail intrinsically might be part of the symmetric kinase domain interacting interface, truncation of part of carboxyl tail causes the receptor conformation change (Endres, Engel et al. 2011). The conserved C-terminal “LVI” motif proximal to kinase domain in EGFR (corresponding to “VVI” in ERBB2) is required for the optimal orientation of receptor kinase domain for transactivation (Schaefer, Akita et al. 1999).
Beyond the regulation of conformation and association, series of studies have shown that EGFR is subjected to post-translational modifications at serine/threonine residues. Thus far, all identified phosphorylation events are inhibitory in nature to receptor signaling. Serine/threonine phosphorylation is also found to increase the efficacy of chemotherapy induced receptor internalization and signaling attenuation (Zwang and Yarden 2006). The kinases responsible for serine/threonine phosphorylation range from CaM Kinase II, cell cycle associated kinase p34\textsuperscript{cdc2}, to stress responsive kinase p38 (Countaway, Nairn et al. 1992, Kuppuswamy, Dalton et al. 1993, Feinmesser, Wicks et al. 1999, Adachi, Natsume et al. 2009). Temporal studies of the serine/threonine phosphorylation of the carboxyl terminal tail of EGFR demonstrate that as a function of EGF stimulation time, the serine/threonine phosphorylation level at specific sites can either stay constant or vary greatly (Wu, Kim et al. 2006). Hence, the carboxyl tail can regulate EGFR function on multiple levels, as a site for tyrosine phosphorylation and adaptor recruitment based ligand transmission as well as serine/threonine phosphorylation based feedback inhibition.

1.4.3 Adaptors/effector proteins binding to the quiescent/unphosphorylated state of receptors

The canonical pathway presented above is largely based on receptor signaling that emanates from activated and phosphorylated ERBB receptors. Yet, adaptor binding to the quiescent or unphosphorylated receptors can also contribute to the control of receptor activation and diversity of signaling outcomes. For example, ERBB2 interaction with Hsp90 helps to stabilize the mature inactive receptor (Xu,
Mimnaugh et al. 2001). Likewise, calmodulin has been found to associate with both purified EGFR and ERBB2 \textit{in vitro}. This juxtamembrane linker mediated binding for EGFR is able to alter the charge composition of the receptor at JM region, as well as change the flexibility of juxtamembrane section (Li, Sánchez-Torres et al. 2004). This conformational change of the receptor in its nonactivated state may alter receptor allosteric activation control.

1.5 The focus of my study and three open questions

The ERBB2-ERBB3 signaling pair represents the most potent signaling pair compared to other ERBB complexes, making ERBB2 an important target in drug development. Although the canonical dimerization-induced activation model has provided a critical foundation for the study of receptor signaling, multiple major pieces are still missing in the big picture. Particularly, as the first ERBB2 targeted antibody therapy by using Herceptin to target ERBB2 has shown inspiring curative effects, its underlying molecular mechanism remains elusive. Indeed, emerging studies have gradually begun to underscore the signaling activation control not only from regions other than ERBB2 ECD and kinase domain, but also from the kinase impaired ERBB3 receptor. Hence, in my thesis work, I have focused on novel modes of ERBB2 activation control involving mechanism from the exterior to the interior of cell membrane.

1.5.1 How is ligand-unbound ERBB2 efficiently phosphorylated in complex with kinase-deficient ERBB3?

Previous studies suggest that the formation of ligand-induced ERBB2-ERBB3 heterodimerization can facilitate receptor activation. However, since ERBB2 does
not bind ligand and ERBB3 is defective in kinase activity, the canonical dimerization model presents challenges in explaining how ERBB2 can be efficiently activated. So far several possibilities have been proposed (Figure 1.8): 1) One recent study by Mark Lemmon’s group shows that ERBB3 is able to specifically incorporate ATP molecules in vitro but the catalytic activity of this reaction is ~1,000 folds lower compared with EGFR. In vitro, ERBB3 kinase activity is also resistant to inhibitors like lapatinib, which is not the case for the observed ERBB2 phosphorylation in a cellular setting (Shi, Telesco et al. 2010). Therefore, it is unlikely that ERBB2 is phosphorylated by the minimal kinase activity of ERBB3. 2) Based on the elucidation of the mechanism of allosteric activation another model has been proposed in which phosphorylation of tyrosine residues at C-terminal tail of ERBB2 is induced by ERBB2 itself in an intracellular manner after its kinase activity is stimulated by allosteric activation in trans (Zhang, Gureasko et al. 2006). However, no data has demonstrated this mechanism up to now. 3) Previous research by Epstein’s group resulted in the proposition that ERBB2 phosphorylation may occur within higher order complexes (HUANG, OUYANG et al. 1998). However, experiment evidence or insight into the nature of this complex has been absent. Thus far, multiple pieces of data have suggested not only the existence but also a specific biological function for the ERBB receptor higher order association. A study by Andrew Clayton shows that EGFR equilibrium on cell surface shifts toward the multimeric forms in presence of ligand and this is associated with promptly increased receptor activation (Clayton, Walker et al. 2005). Of particular
interest, purified ERBB3-ECD is also able to form oligomers (Park, Baron et al. 2008). However, whether these ERBB3 oligomers play a role in signaling activation was unclear. Therefore, in my current study, I characterized the mechanism of ERBB2 activation by using an RNA aptamer A30 selected against the ERBB3 extracellular domain (Zhang, Park et al. 2012). A30 is shown to asymmetrically block the phosphorylation of ERBB2, without impacting phospho-ERBB3 and canonical heterodimerization. From this work, we conclude that higher order complexes of nonoverexpressed ERBB receptors are required for the normal signaling of canonical ERBB2/ERBB3 heterodimer.

Figure 1.8. Proposed models for ERBB2 phosphorylation. A. Phosphorylation of ERBB2 by the minimal ERBB3 kinase activity. B. ERBB2 phosphorylation in cis after allosteric activation by ERBB3 in trans. C. Phosphorylation by other activated ERBB2 receptors within higher order receptor complexes, i.e. proxy activation.

1.5.2 How does serine/threonine phosphorylation at ERBB2 carboxyl terminal tail impact receptor functionality?

The catalytic activity of ERBB receptor can be regulated by itself, in most cases by its juxtamembrane region or carboxyl terminal tail. The juxtamembrane segment is shown to be directly involved in stabilizing the asymmetric dimerization of allosterically activated EGFR kinase domain (Jura, Endres et al. 2009). Crystal structures have already suggested that the tail region proximal to the kinase domain makes extensive contacts with both the N-lobe and C-lobe of
the kinase domain (Endres, Engel et al. 2011). The distal tail region of EGFR and ERBB2 has multiple serine and threonine residues, and it has been found that phosphorylation events at some of those sites are associated with enhanced receptor internalization and decreased tyrosine phosphorylation (Feinmesser, Wicks et al. 1999).

Initially in this project, we wanted to compare the C-terminal tail phosphotyrosine profiles of Chimera ERBB3/2 (ERBB3-ECD fused with ERBB2 TM and cytoplasmic portion) with wild type ERBB2 receptor. The ECD of ERBB3 conveys strong oligomerization to the chimeric receptor, yet with identical cytoplasmic segments and comparable tyrosine phosphorylation levels, signaling outcomes are dramatically different. Unfortunately, we failed to obtain the phosphotyrosine profile since all peptides obtained by in-gel tryptic digestion containing phosphotyrosine residues were too long to yield sufficient sequence coverage. However, multiple serine phosphorylation sites were identified within these ERBB2 carboxyal terminal peptides.

We found that the phosphoserine/threonine modification was associated with inhibited phosphotyrosine status. Preliminary evidence also suggests that p38 might be the potential kinase responsible for phosphorylating these serine clusters at ERBB2 C-terminal tail. Based on similar models for EGFR, we hypothesize that the “fold-back” of the tail region containing phosphorylated serines will establish an inhibited state of the receptor. This investigation not only emphasizes the signaling cross-talk between tumor progression and stress-induced cell response in the network of receptor cascade, but also implies that by
promoting EGFR serine/threonine phosphorylation, the tumor progress might be suppressed.

1.5.3 How does adaptor (e.g. GRB7) recruitment pattern add to the diversity of signaling outcomes?

![Diagram of GRB7 recruitment](image)

Figure 1.9 GRB7 recruitment leads to both cell proliferation and cell migration.

Signaling outcomes depend on not only receptor association and phosphorylation, but also adaptor recruitment. In the process of cell signaling, the final and one of the most crucial step for a receptor is to transduce the extracellular stimuli from cell surface into nucleus. This will involve the recruitment and activation of multiple downstream molecules which can finally result in the transcriptional regulation of target genes. GRB proteins are a family of growth factor receptor bound proteins that were initially identified by screening for downstream targets of EGFR (Margolis, Silvennoinen et al. 1992). GRB7 is a
unique adaptor protein in that it is co-amplified with ERBB2 on chromosome 17q12 (Stein, Wu et al. 1994). That means that every ERBB2 amplified cancer is also GRB7 amplified. Clinically, its RNA overexpression was prominently associated with increased recurrence for triple-negative breast cancer patients treated with chemotherapy (Sparano, Goldstein et al. 2011). Apart from breast cancer, GRB7 is also implicated with gastric cancer, esophageal carcinoma and ovarian carcinogenesis (Han, Shen et al. 2001). As figure 1.9 shows, GRB7 may act as a signaling hub to integrate incoming signals. It may also serve as a molecular scaffold to help assemble signaling complexes, ultimately controlling ERBB2 overexpressing breast cancer progression via tumor cell proliferation and migration (De Pradip, Dey et al. 2013). GRB7 was also shown to exist in an equilibrium between monomer and dimer, with a single point mutation (F511R) being sufficient to disrupt dimers (Porter, Wilce et al. 2005). The dimeric state of GRB7 is intriguing because of the potential signaling consequences in terms of its interaction with receptor clusters. We therefore ask the question how GRB7 is recruited to ERBB2/3 complex mechanistically and how potential differences in recruitment may impact the tumorigenesis of ERBB2. Using coimmunoprecipitation methods, GRB7 is confirmed to be recruited to ERBB2 by two modes: 1) phosphotyrosine dependent binding mediated by its SH2 domain and 2) phosphotyrosine independent binding which is probably mediated by its BPS (Between PH and SH) region.
Altogether, my thesis work provides new perspectives for receptor activation control across the cell membrane. Outside of cell surface, the ERBB3 oligomerization establishes the framework for the formation of receptor higher order structures where ERBB2 became phosphorylated in proxy. By targeting ERBB3 extracellular domain, A30 is able to disrupt a second interaction interface. This leads to its synergistic effects with pertuzumab in inhibiting ERBB2 amplified signaling. In ERBB2 cytoplasmic portion, the clustered ERBB2 may recruit dimerized adaptor GRB7 by dual binding modes, which then creates an additional level of signaling complexity and drug resistance. Importantly, GRB7 may function to integrate FAK and ERBB2 signaling resulting in more potent signaling transduction. On the other hand, activated ERBB2 signaling is attenuated by serine/threonine phosphorylation at receptor C-terminal tail. This negative regulation prevents ERBB2 signaling from causing too much damage to the cell. Most of previously developed drugs to directly target ERBB2 signaling were later on found to cause multiple side effects and to induce strong drug resistance. Our uncovering of novel mechanism of ERBB2/ERBB3 signaling control established fundamental roles for designing new drugs to overcome these obstacles.
Chapter 2

Functional Isolation of Activated and Unilaterally Phosphorylated Heterodimers of ERBB2 and ERBB3 as Scaffolds in Ligand-dependent Signaling

2.1 Summary

The EGFR (ERBB) family provides a model system for receptor signaling, oncogenesis, and the development of targeted therapeutics. Heterodimers of the ligand binding deficient ERBB2 (HER2) receptor and the kinase impaired ERBB3 (HER3) create a potent mitogenic signal, but the phosphorylation of ERBB2 in this context presents a challenge to established models of phosphorylation in trans. Higher order complexes of ERBB receptors have been observed biophysically and offer a theoretical route for ERBB2 phosphorylation, but it is not clear whether such complexes provide functionality beyond the constituent dimers. We now show that a previously selected inhibitory RNA aptamer that targets the extracellular domain (ECD) of ERBB3 acts by sterically disrupting these higher order interactions. Ligand binding, heterodimerization, phosphorylation of ERBB3, and AKT signaling are only minimally affected, whereas ERBB2 phosphorylation and MAPK signaling are selectively inhibited. The mapping of the binding site and creation of aptamer-resistant point mutants are consistent with a model of side-by-side oriented heterodimers to facilitate proxy phosphorylation, even at very low endogenous levels of receptors (below 10,000 receptors per cell). Additional modes of signaling with relevance to pathological ERBB expression states emerge at high receptor levels. Hence,
higher order complexes of nonoverexpressed ERBB receptors are an integral and qualitatively distinct part of normal ERBB2/ERBB3 signaling. This mechanism of activation has implications for models of allosteric control, specificity of interactions, possible mechanisms of cross-talk, and approaches to therapeutic intervention that at present often generate experimental and clinical outcomes that do not reconcile with purely canonical, dimer-based models.

2.2 Background

Biochemical and structural analysis of the EGFR or ERBB (ErbB) family of receptor tyrosine kinases has provided a wealth of molecular details that have contributed significantly to our understanding of cell surface signaling and its deregulation in a broad range of diseases. The longstanding mechanistic model of receptor tyrosine phosphorylation in trans within ligand-activated dimers has undergone significant expansion in recent years. Beyond the regulation at the level of dimers, higher order clustering phenomena have been reported both for inactive and active receptor states (Gadella Jr and Jovin 1995, Nagy, Jenei et al. 1999, Clayton, Walker et al. 2005, Yang, Raymond-Stintz et al. 2007, Clayton, Orchard et al. 2008, Szabó, Horváth et al. 2008, Duke and Graham 2009). However, a critical and so far inaccessible question has been whether higher order complexes create qualitatively distinct signals that cannot emanate from dimers. The functional asymmetry of the closely related ERBB2/ERBB3 heterodimer presents an opportunity for experimental dissection but also a long-standing challenge to existing signaling models. ERBB2 is an orphan receptor that tyrosine phosphorylates its heterodimerization partners. ERBB3 is itself
catalytically impaired but binds ligand, and its kinase domain allosterically activates its partners (Zhang, Gureasko et al. 2006). This functional asymmetry is underscored by the fact that the majority of MAPK signaling emanates from ERBB2, whereas ERBB3 dominates signaling through the PI3K/AKT pathway. Ligand specificity sets neuregulin (NRG)-activated ERBB2/ERBB3 functionally apart from the EGF-activated ERBB2/EGFR. Paradoxically, the neuregulin-dependent activation of ERBB2/ ERBB3 heterodimers results in very efficient phosphorylation of ERBB2, making ERBB2/ERBB3 the most mitogenic receptor pair in the ERBB family (GuY, Platko et al. 1994, Klapper, Glathe et al. 1999). However, the phosphorylation mechanism is not understood. Recent studies have shown that ERBB3 does bind ATP (Jura, Shan et al. 2009, Shi, Telesco et al. 2010) and features a low but specific catalytic activity in vitro (Shi, Telesco et al. 2010). However, the ATP-bound state surprisingly retains a conformation associated with an inactive state (Jura, Shan et al. 2009). The in vitro phosphoryl transfer is very inefficient compared with EGFR and resistant to existing kinase inhibitors of ligand-induced ERBB2/ERBB3 signaling in a cell culture setting (Shi, Telesco et al. 2010). Hence, the primary function of ATP binding by ERBB3 remains an open question. Alternatively, phosphorylation of the C-terminal tail of ERBB2 could conceivably occur in an intramolecular fashion after allosteric activation has occurred in trans. Phosphorylation between activated ERBB2 receptors within higher order complexes, previously termed proxy phosphorylation (HUANG, OUYANG et al. 1998), provides an alternative conceptual framework. Proxy phosphorylation
would presumably involve ligand-activated heterodimers and is therefore mechanistically distinct from the ligand-independent autophosphorylation of overexpressed ERBB2, which is generally thought to involve transient homodimerization. However, whereas various biophysical studies have shown that higher order clusters exist, this hypothesis has so far eluded direct experimental evaluation. More importantly, we so far have no direct evidence that higher order clusters, as detected biophysically for the ERBB receptor family, create unique functionality that goes beyond an assembly of functionally autonomous dimers. A mechanistic understanding of the functional role of higher order complexes was so far limited by our inability to dissect the proxy and canonical dimer component in a molecularly defined manner.

The starting point for our analysis was the inhibition of ligand-induced signaling by A30, a SELEX-derived RNA aptamer against ERBB3 extracellular domains (ECDs). It was characterized initially in vitro and in cell culture with respect to its ERBB3-binding specificity and ability to inhibit NRG-mediated growth stimulation (Chi-hong, Chernis et al. 2003). However, subsequent studies of its mode of inhibition did not reconcile with conventional dimerization models. The present analysis reveals instead that A30 blocks the interactions of activated but functionally nonautonomous heterodimers, thus preventing higher order complexes that are essential for the proxy phosphorylation of ERBB2, even at low endogenous receptor levels. Hence, higher order association and proxy phosphorylation do not only occur, but are in fact indispensable and qualitatively unique components of ERBB2/ERBB3 signaling.
2.3 Materials and Methods

Reagents. Antibodies were obtained from Santa Cruz Biotechnology (ERBB3/C17 and ERBB2/C18), Upstate Biotechnology (pTyr/4G10, pERBB2(Tyr1248), Cell Signaling Technologies [pERBB3(Tyr1289), pMAPK(Thr202/Tyr204), MAPK, pAKT(Ser473), and AKT], Epitomics [pERBB2(Tyr1139), Biogenex (ERBB2/CB11), Invitrogen (V5-HRP and V5), and Evrogen (Dendra)]. The purification of ERBB3–ECD constructs and the Trx–NRG fusion protein of the EGF-like domain of NRG1-β1 with thioredoxin have been described previously (Landgraf and Eisenberg 2000). Pertuzumab was provided by Mark Sliwkowski (Genentech Inc., South San Francisco, CA).

Cell Culture and Transfection. MCF7 cells were maintained in RPMI-1640 [10% (vol/vol) FBS, 5% (vol/vol) CO2]. GFP fusion constructs of ERBB3 were expressed in the pFLAG-MYC-CMV-19 expression vector (Sigma), and cotransfected with puromycin resistance marker. Stably expressing cell lines were sorted for receptor–GFP fusions by FACS before expansion. For transient ERBB3 overexpression, MCF7 cells were transfected with either Den-dra2 (Evrogen) or pFlag-ERBB3-Dendra2.

Aptamer Synthesis. A30 was transcribed as previously described (Chi-hong, Chernis et al. 2003) using the RiboMAX large-scale RNA production system from Promega. For inhibition studies, the minimal aptamer (mA30) was generated using a PCR template with a shortened 5’ end. Synthetic minimal aptamer with a 3’ biotin was used for fluorescence microscopy studies.
Aptamer Inhibition of Ligand Stimulation. Indicated cell lines at 60–70% confluency were treated with aptamer in RNaseOUT supplemented RPMI-1640 for 30 min at 37 °C, stimulated with Trx-NRG (10 nM) for 10 min at room temperature, and lysed in SDS sample buffer (57 mM Tris-HCl, 10% (vol/vol) glycerol, 3.3% (wt/vol) SDS, 0.17 mg/mL bromophenol blue, 1.7 mg/mL DTT) before Western blot analysis with the indicated antibodies. For coimmunoprecipitation, cells were lysed in mild lysis buffer [20 mM Tris, 137 mM NaCl, 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 1× mixture]. Lysates were passed 10 times through a fine-gauge needle and incubated for 5 min at 37 °C. Cell debris was removed by centrifugation at 10,000 × g before immunoprecipitation of supernatants at room temperature for 2 h, five washes in mild lysis buffer and denaturation in 1× SDS sample buffer containing DTT. Inhibition studies in BT474 and SKBr3 cells were carried out as described above for MCF7 cells. For ERBB2 coimmunoprecipitation studies, MCF7 cells were cotransfected with ERBB2 constructs carrying either an N-terminal V5 epitope tag or C-terminal Dendra.

Molecular Modeling. Primary sequences were aligned using clustalW. Structural models were created using Swiss Model builder (Schwede, Kopp et al. 2003) and surface charges were calculated in Swiss viewer using the default parameters. The EGF dimer was used as a structural framework to model the ERBB2/ERBB3 heterodimer. The C terminus of domain IV of ERBB3 (obtained
from the tethered con-formation) was projected into the N-terminal segment of domain IV of EGFR in the dimer structure.

**Live Cell Aptamer Binding Studies.** MCF7 cells with stably overexpressed mutants of ERBB3–GFP were established after FACS sorting for GFP. Cells were dissociated with Versene (PBS/EDTA) and incubated for 10 min with 100 nM 3’ biotinylated minimal A30 and anti-FLAG(M2) antibody (Sigma) followed by 20 min of staining with streptavidin-Texas Red conjugate and Pacific-Blue–labeled secondary. Cells were allowed to settle and images of the uniformly rounded cells were acquired on a Zeiss Axiovert 200 M fluorescence microscope at low magnification in a rapid and automated manner using Openlab command script execution. Following density slicing, cells (<5%) with GFP signal outside two SDs of the mean (mainly due to well boundary effects) were excluded from the analysis. The ratio of surface lo-calization (Pacific Blue) and aptamer binding (Texas Red) was analyzed as discussed. The statistical significance was analyzed using an uncoupled dual distribution t test, with $P < 0.01$ considered significant and $P < 0.001$ very significant.

2.4 Results

2.4.1 The ERBB3-Directed Aptamer Selectively Inhibits Ligand-Induced Phos-phorylation of ERBB2 but Not the Formation of Active Heterodimers

Most available cellular data on ERBB receptors are derived from overexpression systems. This finding reflects both technical reasons and the high importance of receptor amplification in cancer. Comparatively little is known about the rules that govern ERBB signaling at the very low receptor levels found in all but hema-
topoetic cells. Low endogenous ERBB levels play a critical role in the development of several organs and subsequent cardiac maintenance. To better understand both “normal” ERBB signaling and its distortion in a cancer setting, we studied ERBB2/ERBB3 signaling in MCF7 cells, a well established model system for non-ERBB2 overexpressing breast cancers with FACS-confirmed expression levels of both receptors at or below 10,000 receptors per cell. In contrast to autophosphorylation in ERBB2 overexpressing cells, ERBB2/ERBB3 signaling in MCF7 is very sensitive to nanomolar ligand concentrations. The inhibition of ligand binding or receptor heterodimerization is expected to interfere with the activation of both receptors. However, a TyrP immunoprecipitation of ligand-treated samples shows that the lower levels of remaining tyrosine phosphorylation after A30 treatment reside almost exclusively on ERBB3 (Figure 2.1A). For cells with low receptor levels, mild detergent and ligand treatment can significantly influence the recovery of receptors from the membrane compartment. We confirmed the preferential inhibition of ERBB2 phosphorylation by direct SDS lysis and immunoblotting for ERBB2 pTyr1248 (Figure 2.1B), pTyr1139 (e.g., Figure 2.1C), and pTyr1289 for ERBB3. This approach confirms a selective inhibition of ERBB2 phosphorylation. Whereas ERBB2 phosphorylation is almost completely inhibited, ligand-induced coimmunoprecipitation of ERBB2 and ERBB3 is retained as is the phosphorylation of ERBB3 in those complexes (Figure 2.1C). Compared to deliberately partial inhibition by pertuzumab (2C4), an antibody that targets the dimerization loop of ERBB2 and blocks heterodimerization (Agus, Akita et al.
inhibition by A30 is distinctly more asymmetric in nature (Figure 2.1D). This asymmetric inhibition by A30 translates into downstream signaling (Figure 2.2). The inhibition of phosphorylation at ERBB2/Y1139, one of several sites implicated in the initiation of MAPK activation, correlates with a pronounced inhibition of MAPK signaling. This is in contrast to a lack of inhibition for the ERBB3-dependent activation of AKT. Combined, these findings suggest that A30 exerts its primary inhibitory effect at a level other than ligand binding to ERBB3, heterodimerization, or the allosteric activation of ERBB2 in heterodimers. Instead, the asymmetric inhibition of ERBB2 phosphorylation creates an activated heterodimer with equally asymmetric downstream signaling properties.

Figure 2.1. A30 preferentially inhibits ERBB2 phosphorylation but not hetero-dimerization with ERBB3. (A) Inhibition of total tyrosine phosphorylation (Upper) and relative inhibition of ERBB2 and ERRB3 phosphorylation (receptor IB after TyrP-IP under complex disrupting conditions, Lower). (B) A30 inhibition probed directly by site-specific tyrosine phosphorylation (ERBB2/pTyr1248 and ERBB3/ pTyr1289). (C) Coimmunoprecipitation of ERBB2 and ERBB3 and the pERBB3/ (pTyr1289) in recovered complexes are minimally impacted compared with ERBB2 phosphorylation (pTyr1139) inhibition. (D) In contrast to A30, the partial inhibition by the dimerization blocking anti-ERBB2 antibody pertu-zumab (2C4) blocks the phosphorylation of ERBB2 (pTyr1248) and ERBB3 (pTyr1289) proportionally.
2.4.2 Where Does A30 Bind Relative to Known Interacting Surfaces of ERBB Receptors?

A 43-nucleotide minimal aptamer segment (mA30) is sufficient for specific ERBB3 binding and targeted photo cross-linking (Park, Baron et al. 2008). However, whereas mA30 blocks the inhibitory properties of the 78-nucleotide-long A30, it is a much less potent inhibitor of ERBB2 phosphorylation (Figure S2.1). These early observations implicated steric interference with complexes beyond the heterodimer. For an initial approximation of the portion of the ERBB3 ECD involved in A30 binding, we photo-crosslinked thiouracil substituted A30 and insect cell expressed extracellular domains of ERBB3 (Figure S2.2). This in vitro study with purified components narrowed the putative binding region for A30 to a segment near the junction of domain III and IV that is accessible in both the

---

Figure 2.2. The preferential inhibition of ERBB2 correlates with a pronounced inhibition of MAPK activation, whereas both ERBB3 phosphorylation and AKT activation are largely resistant to A30. Note that pERBB3 initially increases at low aptamer concentrations to drop to a plateau of 20% inhibition.
extended and tethered conformation. Because no structure is currently available for the ERBB3 receptor ECD in the extended conformation, we used the available structure of tethered ERBB3 for the initial selection of residues for mutagenesis. A striking feature of the domain III–IV junction is its neutral-to-positive surface charge (Figure 2.3A). Whereas charge-based interactions alone cannot account for the high specificity and nanomolar affinity of A30, electrostatics exert strong “steering” forces during a SELEX procedure. Combined, these considerations further narrowed the list of candidate residues, making an analysis of cell-surface–expressed receptors by mutagenesis feasible. For live cell binding studies, we focused on four locations where mutations were not likely to significantly alter the underlying backbone conformation yet have a significant impact on the immediate surrounding surface. Binding was measured on large numbers of individual live cells using a triple color fluorescent tag assay that quantifies receptor levels (C-terminal GFP fusion, green), surface expression (N-terminal FLAG epitope tag, blue), and aptamer binding (mA30-biotin and streptavidin-Texas Red) on MCF7 cells that stably express the mutant receptors in a large excess over endogenous ERBB3. Figure 2.3B shows the ratio of aptamer binding to surface receptors for 200–300 individual cells. Whereas the removal of the positive surface charge at lysine 453 and arginine 456 results in a modest (10%) increase in A30 binding, the removal of two negative charges at glutamic acid 460 and 461 moderately diminishes binding (7%). Both differences are statistically significant at P < 0.01. More extensive and statistically highly significant inhibition of binding (P < 0.001) was observed after mutating histidines
446/447 (20%) or arginines 471/472 (22%) to alanines. Those four residues form a contiguous surface patch that is spatially close to glutamic acids 460 and 461. H446/H447 represent the C-terminal “cap” of domain III and R471/R472 is located directly in the loop region between domains III and IV. The R471/472 site was selected for charge reversal, resulting in a nearly complete loss of A30 binding (90% inhibition). Inhibition is independent of receptor density (Figure S2.3). The impact of mutagenesis on fully adherent cells is shown in Figure 2.3C, demonstrating the loss of A30 binding to the R471/472E mutant at comparable expression and cell surface presentation levels. The stably expressed R471/472E mutant displays wild-type equivalent responsiveness to ligand, both in terms of ERBB2 tyrosine phosphorylation and down-stream activation of MAPK (Figure 2.4A) but is resistant to inhibition by A30. This response pattern suggests that the mutagenesis impacts A30 binding but is alone insufficient to disrupt proxy phosphorylation.
Figure 2.3 A30 targets the domain III–IV junction region. (A) Location of residues in the tethered conformation of ERBB3 that were selected for mutagenesis on the basis of photo cross-linking data (Fig. S2). Front and back representations of the molecular surfaces are colored by surface charge. (B) The contiguous surface presented by H446/H447 and R471/R472 contributes to A30 binding. Charge inversion at R471/R472, located in the domain III–IV connecting loop, eliminates A30 binding. Measurements represent fluorescence intensity ratios for A30 binding per surface localized receptors within a uniform range of overall receptor levels. Readouts are shown in Fig. 3C. Data (with indicated SDs) are the average for 200–300 individual live cells carrying stably expressed ERBB3 constructs. (C) Representative fluorescence data for clusters of attached live cells showing loss of A30 binding to ERBB3–R471/472E.
2.4.3 Overexpression Shifts the Dynamics of Proxy Phosphorylation

To evaluate whether the mechanism of signaling and inhibition by A30 is sensitive to receptor levels, we transiently overexpressed wild-type ERBB3 in MCF7 cells (Fig 2.4B). Ligand-independent activation is minimally enhanced (measurable only at longer exposures), whereas the ligand-induced phosphorylation of ERBB3 shows both a pronounced increase and enhanced
A30 sensitivity. While the A30 inhibition of ERBB3 phosphorylation at endogenous receptor levels plateaus at 20% (Figure 2.2), the ligand-dependent phosphorylation of overexpressed ERBB3 is suppressed by 60%. By contrast, the level of A30-insensitive phosphorylation is almost constant, regardless of ERBB3 levels. Combined, these inhibitor and ligand responses suggest that upon overexpression, a shift in the mechanism of ERBB3 phosphorylation occurs toward a more A30-sensitive interaction. Whereas levels of ERBB3 that are orders of magnitude higher than ERBB2 are unnatural, the inverse scenario is a hallmark of many ERBB2 overexpressing cancers. We therefore evaluated the impact of A30 and pertuzumab on the ERBB2-overexpressing breast cancer cell line BT474 (Figure 2.4C) and SKBr3 (Figure S2.4), each harboring well over one million ERBB2 receptors in large excess over ERBB3. The phosphorylation of ERBB3 but not ERBB2 is stimulated by ligand, and a pertuzumab concentration that achieves the maximal achievable inhibition (Figure S2.5) is more effective than A30 in suppressing the ligand-induced phosphorylation of ERBB3. Arguably more relevant to the cancer cell scenario, each inhibitor suppresses constitutive ERBB3 phosphorylation only partially, but both act synergistically, resulting in almost complete suppression of constitutive ERBB3 phosphorylation. Whereas largely ineffective in the inhibition of ERBB2 autophosphorylation, A30 is also surprisingly effective in further enhancing the ability of pertuzumab to suppress the constitutive and lapatinib-sensitive (Figure S2.6) phosphorylation of ERBB2.
2.5 Conclusion and Discussion

Our current study of the mode of action of A30 provides insights into the mechanism of ERBB signaling beyond conventional models of functionally autonomous heterodimers. A30 does not interfere with ligand binding to ERBB3, receptor heterodimerization, or AKT signaling. The largely unimpeded tyrosine phosphorylation of ERBB3, even within coimmunoprecipitated heterodimers, indicates that the allosteric activation of the kinase activity of ERBB2 by ERBB3 is also not inhibited. However, under these conditions, ERBB2 tyrosine phosphorylation and much of the associated MAPK signaling is inhibited. Hence, aptamer binding creates the heterodimers that one would have theoretically expected on the basis of the impaired kinase activity of ERBB3 and a mechanism of transphosphorylation.

For the ERBB2/ERBB3 system, both EM (Yang, Raymond-Stintz et al. 2007) and homo-FRET correlated anisotropy measurements (Szabó, Horváth et al. 2008) indicate that rather than a formation of dimers from monomers, activation involves a rearrangement of clusters. Depending on the receptor species, clusters may be defined by proximity or, as in the case of ligand-free ERBB3, through actual oligomerization. For EGFR, independent studies have demonstrated the ligand-dependent emergence of tetramers as a dominant species in a population (Clayton, Walker et al. 2005). However, the mechanistic contribution of higher order complexes has so far been unclear, and studies have been limited to high expression levels out of necessity. Assuming catalytically fully inactive ERBB3, Epstein and colleagues proposed proxy phosphorylation of
ERBB2 in the past as a logical conclusion on the basis of the understanding of the individual receptor properties (HUANG, OUYANG et al. 1998). However, a study of the intermediate building blocks had been out of reach. The current study uniquely demonstrates through inhibitor-driven experimental dissection, that self-standing ERBB2/ERBB3 heterodimers constitute activated signaling units, but with signaling properties that are imbalanced with regard to the classic, two-legged MAPK and AKT signaling that is a well-established hallmark of ERBB2/ERBB3 activation in a cellular setting. Hence, higher order complexes do not only exist for ERBB2/ERBB3, they are a basic mechanistic component needed to qualitatively create the ERBB2/ERBB3 signaling as we know it. Furthermore, these secondary inter-actions are crucial for normal signaling, even at low endogenous receptor levels that have so far been out of reach for the direct biophysical studies that first reported on the existence of higher order clusters.

What is the nature of the higher order complex? Our study was deliberately aimed at the nature of normal, ligand-dependent ERBB signaling at low endogenous receptor levels. Whereas the exact stoichiometry of the relevant higher order complexes is not readily accessible at low endogenous levels on technical grounds, we can address whether the mapping of the aptamer binding site unto the heterodimer is consistent with specific models derived from EGFR at high expression levels. For EGFR, FRET distance measurements suggest a poorly understood dependency of higher order interactions on the cellular context (Whitson, Beechem et al. 2004) and a likely side-by-side orientation of canonical
dimers to form transient tetramers (Clayton, Orchard et al. 2008). To interrogate whether our inhibition data are consistent with such a side-by-side arrangement of heterodimers, we mapped the binding site of A30 on ERBB3. The residues that convey aptamer resistance are located distant from both the ligand-binding site and the canonical dimerization interface when mapped onto the crystallographically available tethered conformation. Within a homology model of the ERBB2/ERBB3 heterodimer, based on the crystal structure of ligand-bound dimeric EGFR ECDs (Garrett, McKern et al. 2002, Ogiso, Ishitani et al. 2002), the A30 binding site is located at the edge of a surface on ERBB3 with a distinctly neutral-to-positive surface charge. This patch stands out against the negative surface charge of ERBB2 and the remainder of ERBB3 (Figure 2.5A). Within a side-by-side arrangement, such a charge distribution would favor transient interactions between dimers (Figure 2.5B). A minimal version of A30 binds ERBB3 with high affinity but poorly inhibits ERBB2 phosphorylation. The tail region of full-length A30, which does not contribute to binding but almost doubles the size of the aptamer, confers a strong increase in inhibitory potency (Figure S2.1). Hence, the mapping of the A30 binding site and functional inhibition data are consistent with a molecular model of side-by-side oriented heterodimers as the next, but not necessarily final level in the receptor assembly.

Whereas ERBB2 alone does not bind ligand, the proposed model predicts a ligand-dependent increase in higher order complexes that contain two ERBB2 receptors. Although the proposed tetrameric state is expected to be relatively unstable, we tested the ability to coimmunoprecipitate two differentially tag-ged
species of ERBB2 in a ligand-dependent but A30-sensitive manner. While heterodimers are readily recovered in CoIPs (e.g. Figure 2.1C), the recovery of coimmunoprecipitated ERBB2 species is low. Nevertheless, we observed a significant ligand-dependent increase in coimmunoprecipitated Dendra and V5-tagged ERBB2 species (Figure 2.5C). This CoIP was suppressed by the addition of A30. This observation matches data in a recent study by Ghosh et al. on the disruption of ERBB2 homodimers by trastuzumab (Ghosh, Narasanna et al. 2011). Whereas the authors used AP1510-mediated dimerization of ERBB2–FKBP fusion proteins, “negative” controls using NRG stimulation instead did not match AP1510 in forced dimerization but detected a 60% increase of ERBB2–ERBB2-containing complexes based on fluorescence proximity assays.

Figure 2.5. Model for receptor phosphorylation in higher order complex (A) Homology model of the ERBB2/ERBB3 heterodimer places the A30 interface on the side of the ERBB2/ERBB3 heterodimer, distant from the canonical dimerization interface and on the edge of a unique positively charged surface patch within ERBB3. (B) Cartoon of the heterodimer in the plasma membrane (PM) with indicated A30-binding site and charge complimentary interface on ERBB3 in dark blue. Cytoplasmic components are not drawn to scale. The structure-derived cartoon outline for the Top view highlights the interlocking canonical dimer interface, the charge complimentary interface on ERBB3 (blue), and the binding sites for A30 and NRG. (C) Ligand-enhanced coimmunoprecipitation of overexpressed V5 and Dendra tagged ERBB2 in the presence of low endogenous ERBB3 in MCF7 cells. The V5 epitope tag was immunoprecipitated. Ligand or A30 were added as indicated. (D) Proposed modes of receptor interactions and impact of A30 at low endogenous receptor levels compared with ERBB3 overexpression. Arrows indicate the directionality of tyrosine phosphorylation with arrow thickness indicating relative contributions.
Our primary model system for biochemical dissection was MCF7 cells, which are often classified as ERBB2 negative by cancer overexpression standards. The mode of signaling we describe appears primarily geared toward maximizing activation at the low receptor levels present in most tissues. Upon overexpression, the mechanism of signaling appears to change qualitatively, and a second mode of activation emerges that uses both interfaces in a dimerization partner-independent manner (Fig. 4B). For overexpressed ERBB3, this mode of phosphorylation stands out through its sensitivity to A30 and strict dependency on ligand, suggesting that it makes use of interactions with a pre-assembled and preactivated heterodimeric scaffold. Fig. 5D compares the proposed flow of phosphorylation under conditions of balanced receptor levels versus overexpressed ERBB3.

A model in which ERBB2 can use two alternative interfaces for signaling also matches a comparative study of the ERBB2-directed, therapeutic antibodies pertuzumab and trastuzumab (Herceptin). It has long been known that both antibodies are not redundant but synergistic in targeting overexpressed ERBB2. Whereas trastuzumab is inefficient in interfering with ligand-induced heterodimerization (Agus, Akita et al. 2002), it is surprisingly more efficient than pertuzumab in blocking constitutive ERBB3 phosphorylation (Junttila, Akita et al. 2009). The large size of Herceptin and the spatially flexible nature of the segment of domain IV that it targets limited the mechanistic exploration of this observation. Our observed synergy of pertuzumab and A30 would involve A30 targeting the secondary interface in a similar manner than Herceptin, except on
the side of ERBB3. In addition, A30 is a much smaller reagent that binds to a region of the ERBB3 receptor for which the placement in the receptor dimer is structurally definable by homology modeling. Interestingly, A30 amplified the ability of pertuzumab to block constitutive ERBB2 phosphorylation. This may suggest that at high levels of ERBB2, ERBB3 may not only be a target of constitutive phosphorylation and driver for enhanced cancer cell survival. Instead it may also serve ligand independently as a scaffold that facilitates efficient auto-phosphorylation of ERBB2 through two alternative approaches. Thus, whereas the primary objective of our study was the dissection of normal ERBB2/ERBB3 signaling, it has direct applicability to the distortion of ERBB signaling that results from overexpression and that cannot readily be explained within the confines of the canonical dimer model.

Regardless of whether tetramers are an endpoint or an intermediate for higher order complexes, it is important to realize that such association states are in competition with other states of clustering or oligomerization. Whereas ERBB2 does not form stable homooligomers, homo-FRET correlated anisotropy measurements indicate that it is organized in spatial clusters at high expression levels, and the average cluster size decreases upon addition of ligand, whereas the opposite appears true for EGFR (Szabó, Horváth et al. 2008). In the absence of ligand, ERBB3 is unique in its tendency to form actual oligomers that sequester the receptor away from its heterodimerization partners, and ERBB3 directed aptamers destabilize ERBB3 homooligomerization (Park, Baron et al. 2008).
The lack of functional autonomy of the heterodimer does challenge our existing model for the regulation of ERBB receptors. What are the determinants of specificity in signal propagation once a ligand-bound dimer has established a preactivated interaction scaffold? Does allosteric kinase domain activation occur between dimers? Do allosteric acceptor and donor kinase domains trade places in “symmetric” EGFR dimers, or does a dedicated acceptor kinase domain need proxy phosphorylation in the reported tetrameric states to become phosphorylated itself? These questions require that the receptors in their entirety, including cytoplasmic kinase domain interactions, are integrated into more dynamic models of complex formation. Already existing data on clinical antibodies suggest that such expanded models may help in demystifying many of the observed and clinically highly relevant inconsistencies with traditional dimer-based models of signaling.
Supplementary Figures

Figure S2.1. Efficient inhibition of ERBB2 phosphorylation (Tyr1139) by A30 relies on the steric interference by its nonbinding tail region (A) A30 (78 nt) has increased potency as an inhibitor of NRG-induced ERBB2 tyrosine phosphorylation compared with its binding competent, minimized counterpart (mA30, 43 nt). (B) mA30 represents the core of the SELEX-derived aptamer with its ERBB3-specific binding region but without the nonbinding, and vector-derived 5′ and 3′ regions. (C) Whereas mA30 fails to inhibit ERBB2 phosphorylation, a molar excess suppresses the inhibitory action of the larger, nonminimized aptamer.
Figure S2.2. A30 targets the junction of domains III and IV in the ERBB3 ECD but not the dimerization interface, and A30 is not in competition with NRG. (A) SDS/PAGE analysis of the photo cross-linking of thiouracil substituted A30 (AT) and nonsubstituted controls (A) to various insect cell-expressed recombinant ECD constructs of ERBB3. ECDs were visualized by Western blotting of their C-terminal V5 epitope tag. Constitutively tethered or extended variants are based on previously described disulfide bond and tether mutations. Domain exchanges with sections of ERBB2 indicated that an intact domain III–IV junction is needed for A30 binding. B2(IV) denotes that domains I–III are derived from ERBB3 and domain IV is derived from ERBB2. (B) Cartoon representation of ERBB3 ECD domains and alternative tethered and extended conformations. (C) Summary of constructs and putative binding site. The ability of the constructs to cross-link to A30, with or without NRG, is indicated as + or −, respectively. (D) Extracellular domain IV swapped constructs of ERBB2 and ERBB3 were combined with the transmembrane and cytoplasmic portions of ERBB2 to create signaling competent receptors, transfected into CHO cells and stimulated with 10 nM NRG or 10 min as indicated. B2(IV) contains domains I–III of ERBB3 and constitutes a fully ligand responsive chimera. B2(I–III)-based chimera effectively represent ERBB2 containing only domain IV from ERBB3. This construct is not ligand responsive in isolation but capable of heterodimerization and ligand-dependent activation when cotransfected with ERBB3. Chimera levels were evaluated with the C18 antibody (Santa Cruz), which targets the cytoplasmic tail of ERBB2. The tyrosine phosphorylation response was measured using the nonresidue-specific 4G10 pTyr antibody.
Figure S2.3. (A) A30 binding and its loss in ERBB3 mutants is independent of receptor density. Data represent individual single cell measurements of surface bound A30 fluorescence for wild type (blue), R471A/R472A (green), and R471E/R472E (red) mutants as a function of surface receptor levels.

Figure S2.4. Inhibition of constitutive and ligand-induced receptor phosphorylation in SKBr3 breast cancer cells. Cells were pretreated with the indicated inhibitors (25μg/mL 2C4 or 100 nM A30) for 30 min followed by 10 min of ligand stimulation (10 nM NRG). ERBB2 phosphorylation was evaluated at Tyr1139 and ERBB3 at Tyr1289. The experimental outline and interpretation match that of the inhibition of BT474 cells as discussed for Fig. 4C.

Figure S2.5. The observed inhibition exerted by pertuzumab (2C4) is not limited by antibody concentration. Under the assay conditions described for Fig. 4C and Fig. S4, the constitutive tyrosine phosphorylation of ERBB2 and ERBB3 was evaluated after treatment with either 2.5 or 25 μg/mL 2C4. A 10-fold increase in 2C4 concentration did not yield any further increase in inhibition.
Figure S2.6. Phosphorylation of ERBB2 at Tyr1139 is correlated but not critical for efficient MAPK activation. In addition, constitutive tyrosine phosphorylation of ERBB2 is very inefficient, compared with ligand stimulation, in triggering MAPK phosphorylation. (A) MCF7 cells carrying low endogenous levels of ERBB2 and ERBB3 were transfected with wild-type or Y1139F mutant ERBB2 carrying an N-terminal FLAG epitope tag. Basal MAPK activation is increased in both cases but the level of activation derived from constitutive ERBB2 phosphorylation is small compared with the MAPK activation induced by ligand through the very limited number of ERBB2/ERBB3 complexes. (B) Ligand-dependent activation of endogenous ERBB2 requires up to 20× more material to be analyzed by Western blot compared with ERBB2 overexpressing cells (reflecting the 10,000 to >1 million ratio in receptors per cell). Ligand-induced MAPK activation correlates with the phosphorylation of Tyr1139. Although ERBB2(Y1139F) is expected to dominate in ERBB2/ERBB3 heterodimers by more than 100:1, no significant impact is observed in the ligand-dependent activation of MAPK. This finding is consistent with the redundancy of tyrosine phosphorylation sites on ERBB2 known to signal into the MAPK cascade combined with the highly amplified activation in the context of heterodimers. The latter requires only a small proportion of activated heterodimers for robust activation. (C) The pY1139 antibody is highly selective. The very low signal obtained for ligand-stimulated and Y1139F mutant overexpressing MCF7 cells is not derived from the mutant receptor and likely represents the low levels of endogenous ERBB2 that are phosphorylated by Y1139F. To demonstrate this point, both wild-type and mutant recombinant ERBB2 were C-terminally biotin tagged (and cotransfected with BirA biotinyl transferase), enriched on neutravidin beads in the presence of 2 M urea, and probed for ERBB2(pTyr1139). Under those conditions, the wild type but not the Y1139F mutant probes positive for tyrosine phosphorylation at Tyr1139. (D) The constitutive phosphorylation of ERBB2 in SKBR3 cells and BT474 that is detected by the pY1139 antibody is fully blocked by the ERBB inhibitor lapatinib.
Chapter 3

p38 Mediated Serine Phosphorylation of ERBB2 at Receptor Carboxyl Tail is Inhibitory to ERBB2 Tyrosine Phosphorylation

3.1 Summary

As the extracellular region and kinase domain play central roles in controlling receptor activation, other portions of ERBB receptor, such as the juxtamembrane region and unstructured carboxyl terminal tail, are also important in regulating the activity of RTKs. The juxtamembrane segment is directly involved in stabilizing the asymmetric dimerization of activated EGFR kinase domain. Protein sequence alignment shows multiple consensus serine/threonine for EGFR and ERBB2 at carboxyl terminal tail. For EGFR, phosphorylation at these residues is implicated with inhibited receptor kinase activity, increased receptor internalization and degradation, as well as reduced cell proliferation. Nevertheless, for the clinically more relevant ERBB2 receptor, a study of the role of serine/threonine phosphorylation at its tail region is lacking. In the current study, mass spectrometry gives us some information regarding ERBB2 serine phosphorylation at the receptor carboxyl terminal tail. By replacing these serines with alanines, the receptor tyrosine phosphorylation level is elevated, suggesting that serine modification is inhibitory to receptor signaling. Among these serines, S1078 is shown as the most crucial single mutation sufficient to promote a two-fold increase in phosphotyrosine level. Preliminary transfection data indicate constitutively activated p38 may mediate the phosphorylation of serines at ERBB2 carboxyl terminal tail. We propose that the tail region with phosphorylated serines will interact with receptor kinase domain
and create steric hindrance to inhibit receptor kinase activity. Further studies such as in vitro kinase assay need to be done to verify these results. All together, our research presents the molecular mechanism for signaling cross-talk between cell stress mediated p38 response and ERBB2 induced cancer progression.

3.2 Background

Multiple serine/threonine modifications at the EGFR C-terminal tail have been reported in the last two decades. Gordon N. Gill showed that EGFR was phosphorylated at Serine 1046/1047 and this phosphorylation requires receptor tyrosine kinase activity (Heisermann and Gill 1988). More detailed studies reveal calmodulin-dependent protein kinase II (CaM Kinase II) is responsible for phosphorylating EGFR at Ser 1046/1047 in vitro, which was associated with receptor densensitization, inhibition of EGFR tyrosine kinase activity, and decrease of MAP kinase activity (Heisermann and Gill 1988, Countaway, Nairn et al. 1992, Theroux, Latour et al. 1992). Thus, Ser1046/1047 phosphorylation may play a negative regulatory role that may inhibit EGFR oncogenic potential. Several mechanisms have been proposed for phosphor-Ser1046/1047 mediated inhibition of EGFR kinase activity. 1) CaM Kinase II may inhibit EGF binding and/or receptor dimerization (Theroux, Latour et al. 1992). However, no marked effect of CaM kinase II on EGF binding or receptor dimerization was observed in experiments using covalent cross-linking analysis (Theroux, Latour et al. 1992).

2) The phosphorylation of Ser1046/1047 may alter the EGFR conformation (Buchdunger, Cioffi et al. 2000). 3) Tyrosine phosphatases sensitive to
serine/threonine phosphorylation of the receptor are recruited to the receptor (Dudley, Pang et al. 1995).

New CaM Kinase II phosphorylation sites were identified in the cytoplasmic tail of EGFR, which were located within a consensus sequence of -S-X-D-. Site-specific mutants of serine 1057 and serine 1142 to alanines were found to potentiate the increased NIH3T3 fibroblast transformation and up-regulated receptor autokinase activity induced by Ser1046/1047 mutations (Feinmesser, Wicks et al. 1999). The desensitization of EGFR upon EGF stimulation is associated with phosphorylation of Serine 1002 as well. A cross-species study was performed due to the availability of materials. In this experiment, purified Xenopus p34\textsuperscript{cdc2} can phosphorylate both the synthetic peptide and purified EGFR from A431 cells at Ser 1002, correlating with a decrease in protein tyrosine kinase activity. These data provides evidence that the EGF receptor may be a target for phosphorylation by a cyclin-dependent kinase \textit{in vivo} and imply that receptor function may be regulated in a cell cycle-dependent fashion (Kuppuswamy, Dalton et al. 1993).

For ERBB2, the wild-type receptor becomes phosphorylated at Ser 1113 following treatment of NIH3T3 cells with growth factors or tyrosine phosphatase inhibitors (Ouyang, Gulliford et al. 2001). In addition, threonine 1172 of ERBB2 can be phosphorylated by CaM K II both \textit{in vivo} and \textit{in vitro}. When overexpressed, both EGFR and ERBB2 with threonine to alanine mutation at residue 1172 showed a defect in receptor desensitisation and underwent a more sustained EGF-induced receptor autophosphorylation compared to wild-type
(Feinmesser, Gray et al. 1996). All these previously discovered serine/threonine phosphorylation sites on EGFR or ERBB2 carboxyl terminal tail and the kinases responsible for phosphorylation are summarized in table 1.

<table>
<thead>
<tr>
<th>Site</th>
<th>Enzymes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR-Serine 1046/7</td>
<td>CaM Kinase II</td>
<td>Gary JH. 1988; Janice LC. 1992</td>
</tr>
<tr>
<td>EGFR-Serine 1057</td>
<td>CaM Kinase II</td>
<td>Rachel LF 1999</td>
</tr>
<tr>
<td>EGFR Serine 1142</td>
<td>CaM Kinase II</td>
<td>Rachel FL 1999</td>
</tr>
<tr>
<td>EGFR-Serine 1002</td>
<td>p34&lt;sup&gt;cr2&lt;/sup&gt;</td>
<td>Dhandapani K 1993.</td>
</tr>
<tr>
<td>EGFR 1002-1022</td>
<td>p38</td>
<td>Yaara Zwang. 2006</td>
</tr>
<tr>
<td>ERBB2-Threonine 1172</td>
<td>CaM Kinase II</td>
<td>Feinmesser RL. 1996</td>
</tr>
<tr>
<td>ERBB2-Serine 1113</td>
<td>Unknown</td>
<td>Ouyang 2001</td>
</tr>
</tbody>
</table>

Table 1. Serine/Threonine phosphorylation sites identified at EGFR and ERBB2 carboxyl terminal tail.

In this study, mass spectrometry results suggested multiple potential serine phosphorylation sites at ERBB2 carboxyl terminal tail, while we identified only one site related to the regulation of receptor function. These serine phosphorylation events were correlated with suppressed receptor tyrosine phosphorylation. Preliminary result identified p38 as the potential kinase to mediate the phosphorylation of these serine residues.

3.3 Materials and Methods

Reagents

Antibodies and reagents were obtained as follow: goat anti-rabbit HRP, and Protein A/G PLUS Agarose from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phosphotyrosine (4G10 and conjugates) from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-p-ERBB2 (Tyr1139 #1991-1) from Epitomics, Inc.
(Burlingame, CA); anti-ERBB2 (CB11) from Biogenex Inc. (San Ramon, CA); anti-Tubulin from ABcam Inc. (Cambridge, MA); anti-pFlag from Sigma-Aldrich (St. Louis, MO). Lipofectamine and plus reagent for cell transfection were purchased from Invitrogen Inc (Carlsbad, CA).

**Cell culture and transfection**

MCF7 cells were maintained in RPMI-1640 medium (Mediatech) supplemented with 10% fetal bovine serum (Gemini Bio) at 37°C and 5% CO2. pFlag-ERBB2 wild type and mutants were transfected into MCF7 cells using lipofectamine and cells were lysed 36 hours after transfection.

**Cloning and site-directed mutagenesis**

ERBB2 cytoplasmic domain was cloned from pFlag-c-myc vector into PUC18 using forward primer: 5’-CGTATCGTGAAAGCTTTCCGGAAGTACACG ATG-3, and reverse primer: 5’-TGCCAGTGGGTATCGATTAATCTAGAGC-3’. Serines identified to be phosphorylated were mutated to alanine or glutamic acid within PUC 18 vector using site-directed mutagenesis kit from Agilent (The primers are attached in Table II). The mutants were sequenced and subcloned back into pFlag mammalian expression vector. For *E. coli* expression, peptides containing different combinations of serines were cloned into pQE40 vector using forward primer:

5’CCGCCGCGCCGGAATTCAATTAAAGAGGAGAAATTAATATGTGCGAGGCCC CCAGG3’

Reverse primers:

5’-GGAGGAGGAGACCGTGGTCTGCACCATCAATACACATC-3’;
5'-GGAGGAGGAGACCGGTAGATCTGTAGCCATCAGTCTC-3’.

Western Blot, Immunoprecipitation and Biotin Pull Down Assay

MCF7 cells at the confluency of 60-70% were transfected with pFlag-ERBB2 WT or serine-mutants. Alternatively, pFlag-ERBB2-Dendra2-Biotin WT or serine mutated constructs were transfected together with pLXSN-BirA vector into MCF7. 36 hours after transfection, cells were lysed in mild lysis buffer (20mM Tris, 137mM NaCl, 1% Triton X-100, 10% glycerol, 5mM EDTA, 1mM sodium orthovanadate, 1mM PMSF, 1X cocktail). Lysates were passed through a fine gauge needle for ten times, and incubated for 5 min at 37°C. Cells debris was removed by centrifugation at 10,000g for 15 min. Protein A/G PLUS agrose mixed with anti-pFlag antibody (Neutravidin beads for Biotin Pull Down) were incubated with cell lysates for 2h at room temperature. The immonoprecipitates were washed five times in Mild Lysis Buffer and boiled for 5 min in 30μL 1X SDS sample buffer containing DTT.

3.4 Results

3.4.1 Multiple serine/threonine phosphorylation sites were identified at ERBB2 carboxyal terminal tail and they were associated with the receptor tyrosine phosphorylation level

Initially in this project, we wanted to compare the C-terminal tail phosphotyrosine profiles of Chimera ERBB3/2 (ERBB3-ECD fused with ERBB2 TM and cytoplasmic portion) with wild type ERBB2 receptor. The ECD of chimera receptor exists in oligomeric state whereas ERBB2 does not. With identical cytoplasmic segments and comparable tyrosine phosphorylation levels, signaling
outcomes for ERBB2 and chimera receptors are dramatically different. So we did the phosphotyrosine IP for HER2 transiently transfectly MCF7 cells and after this, we sent samples to LC/MS-MS analysis. Unfortunately, we failed to obtain the phosphotyrosine profile since all the fragmented peptides containing pTyr residues were too long to fly in the field. However, multiple potential serine phosphorylation sites were identified within these ERBB2 carboxyal terminal peptides. (Figure 3.1).

To approximately screen which serines play a major role in regulating ERBB2 function, we mutated the serines to alanines in different random combinations to cover as many sites and redundancy as possible and evaluated phospho-ERBB2
level as the readout. We selected serine residues that also exist in EGFR at the homologous sites as serines/threonines, or its constitutively activated mimic residue glutamic acid. As shown in Figure 3.2A and B, transient overexpression of pFlag-ERBB2 plasmid bearing combined serine mutations (D, E, F and G) into MCF7 cells upregulates the tyrosine phosphorylation level of ERBB2 at PY1139, the major site responsible for GRB2 adaptor recruitment and MAP Kinase activation. By comparing mutants D, E, F, G with A, B, C, we conclude that the elevated tyrosine phosphorylation might be mainly induced by mutations of residues S1078, S1107 and S1113.

Figure 3.2. Serine to Alanine mutation at the carboxyl tail of ERBB2 elevates ERBB2 tyrosine phosphorylation. Cell lysates from MCF7 transiently transfected with pFlag-ERBB2 serine mutants were evaluated for their relative p-ERBB2 (PY1139)/pFlag level. The results indicated that constitutively inactive mutations will lead to enhanced ERBB2 tyrosine phosphorylation. By comparing highly tyrosine phosphorylated mutants D,E,F with those unaffected ones or WT, it is conclusive that residues S1078, S1107, S1113 may be crucial for the regulation.
To evaluate the contribution of individual residues, I did both serine to alanine and to glutamic acid mutations at residues S1078, S1107, S1113. The relative phospho-ERBB2 level of mutant S1078 shows a 20% increase in relative ERBB2 tyrosine phosphorylation compared to wild type when cell lysates were directly probed using a phospho-ERBB2 antibody (Figure 3.3A and reproduced data not shown). Theroux SJ (Theroux, Latour et al. 1992) and Feinmesser RL (Feinmesser, Wicks et al. 1999) measured EGFR autophosphorylation levels by isolating exogenous receptors using immunoprecipitation. Using the same method, the S1078A mutant shows a 2-3 fold increase of both site-specific (pY1139) and total tyrosine phosphorylation of ERBB2 after Flag immunoprecipitation of cell lysates. The difference in phospho-ERBB2 increase
for S1078A between Figure 3.3 A and 3.3.B is qualitative but not quantitative. However, the phospho-tyrosine status of all glutamic acid mutants did not change much compared to wild type. As a Dendra2-Biotin tag is attached to the carboxyl terminal of S1078A and S1078E mutants, the increase of receptor relative tyrosine phosphorylation for the Dendra attached mutant is higher than that for the one without this tag.

3.4.2 p38 might be the kinase responsible for phosphorylating multiple serine residues at ERBB2 carboxyl tail

Y.Yarden and colleagues have shown that phosphorylation of EGFR at a short segment (amino acids 1002-1022) is carried out by p38 MAP kinase (Zwang and Yarden 2006). Sequence alignment indicates several of our identified key serine phosphorylation residues at ERBB2 C-tail to fall into the homologous sequence of ERBB2. Therefore, it is conceivable that these serine residues at ERBB2 carboxyl terminal tail might also be phosphorylated by p38. As an initial evaluation of the involvement of p38, we activated or deactivated p38 by transfecting either MKK3(Glu), MKK6(Glu), or p38KD into MCF7 cells. MKK3(Glu) represents dominant-active mutant by replacing Ser189 /Thr193 with glutamic acid residues, and a similar strategy is used to replace Ser207/Thr211 with Glu to create MKK6(Glu). (Raingeaud, Whitmarsh et al. 1996) As Thr180 and Tyr182 are changed to Ala and Phe respectively, p38 becomes a dominant-negative mutant with diminished kinase activity (Enslen, Raingeaud et al. 1998). In figure 3.4, transfection of MKK3 or MKK6, which is expected to constitutively activate p38, resulted in the inhibition of wild type ERBB2 tyrosine phosphorylation. This
suggests inhibitory effect on receptor tyrosine phosphorylation might be mediated by p38. However, as mutant #E, which in the previous experiment showed the most significant phosphotyrosine increase, is transfected, the inhibition was removed, suggesting that these serines mutated in #E might be required to mediate the inhibitory effect of p38 activation.

![Figure 3.4. Testing of p38 as a potential kinase to phosphorylate serine residues at ERBB2 carboxyl tail. MKK3(Glu) and MKK6(Glu) can inhibit relative receptor tyrosine phosphorylation in the wild type but not S→E mutant transfected cell lines. From left to right: MCF7 cells transfected with Flag-ERBB2 wild type only, or in combination with MKK3(Glu), MKK6(Glu), and p38KD respectively, followed by MCF7 transfected with S→E mutant (corresponding to mutant E in figure 2) only, or in combination with MKK3(Glu), MKK6(Glu), and p38KD respectively.]

3.5 Discussion

We have identified multiple serine phosphorylation sites at ERBB2 carboxyl terminus and verified their inhibitory effect on ERBB2 phosphotyrosine. p38
might be the candidate kinase to carry out this serine phosphorylation event. Site-directed mutagenesis and immunoprecipitation screening assays have confirmed that S1078 plays an important role in suppressing the tyrosine phosphorylation of ERBB2 by ~2-3 folds (the same extent reported for EGFR serine mutants).

Based on the above results and available model for EGFR (Feinmesser, Wicks et al. 1999), we propose a mechanism for the phosphorylated serines to inhibit ERBB2 receptor allosteric activation. As shown on the right panel of figure 3.5, dominant active MKK will constitutively active p38, resulting in phosphorylation of serine residues at ERBB2 C-terminal tail. The carboxyl terminal tail with phosphorylated serines will inhibit receptor tyrosine kinase activity, leading to observed inhibition of receptor phosphotyrosine. On the left panel, when serines at ERBB2 carboxyl terminus are unphosphorylated, ERBB2 kinase activity is not suppressed by the fold back mechanism. Hence, the tyrosines of the receptor are phosphorylated in proxy.

Our data suggest that p38 might be the candidate kinase phosphorylating ERBB2 serine residues at C-terminal tail. In figure 3.4, while both constitutively active form of MKK3 and MKK3 resulted in suppressed tyrosine phosphorylation of ERBB2, MKK6 active form was more potent. MKK6 is a common activator of p38α, p38β2, and p38γ MAP kinase isoforms, whereas MKK3 activates only p38α and p38γ MAP kinase isoforms (Enslen, Raingeaud et al. 1998). Thereby, MKK6 has a wider range of p38 substrates, which may explain why it is more potent in inhibiting ERBB2 tyrosine phosphorylation. This also indicates that the
p38β2 isoform might be the strongest isoform to mediate the attenuation of EBRB2 tyrosine phosphorylation. Our results also showed that transfection with constitutively active MKK significantly destabilized ERBB2 receptor. The underlying mechanism awaits further investigation.

The inability of S1078E mutant to induce elevated receptor tyrosine phosphorylation compared to wild type ERBB2 might be due to the following reasons: 1) The glutamic acid failed to mimic the constitutive phosphorylation of serine. If this is true, then the glutamic acid mutants should have given the same readout as the alanine mutants, which is not the case in our results. 2) The S1078E mutant successfully mimics serine phosphorylation but the wild type...
ERBB2 receptor is already constitutively phosphorylated at serine 1078. Therefore, different tyrosine phosphorylation levels should not be distinguished between wild type and the mutated ERBB2. 3) Although the mutant S1078A at single residue can lead to increased ERBB2 phosphotyrosine, multiple serine residues may need to be replaced by glutamic acids in order to induce the opposite effect.

Altogether, our finding that multiple serine residues at carboxyl terminal tail of ERBB2 are targets of p38 suggests that the stress signaling stimulated by overexpression of ERBB2 can negatively regulate receptor function through a feedback mechanism.
Chapter 4

**GRB7 is Recruited to ERBB2 through Two Distinct Modes: Phosphotyrosine Dependent Binding Mediated by GRB7-SH2 Domain and Phosphotyrosine Independent Binding Probably Mediated by GRB7-BPS Region**

### 4.1 Summary

Phosphorylated ERBB receptors are able to recruit various downstream adaptor molecules or enzymes to further activate signaling transduction cascades. GRB7 has been identified as such an effector protein downstream of the ERBB2 receptor. The *GRB7* gene is located adjacent to ERBB2 on chromosome 17q12 and hence is coamplified in all cases of *ERBB2* gene amplification. It is implicated in breast cancer, gastric cancer, esophageal carcinoma and ovarian carcinogenesis through the activation of cell proliferation and migration pathways. Conventionally, the interaction of this protein with ERBB2 was shown to be mediated by the Src homology 2 (SH2) domain of GRB7 in a receptor phosphotyrosine dependent manner.

Here we demonstrate the existence of a second novel binding mode which is independent of both the GRB7-SH2 domain and ERBB2 receptor tyrosine phosphorylation. However, compared to ERBB2, GRB7 can only bind to phosphorylated ERBB3. This multi-domain binding mode of GRB7 to ERBB2/3 receptors may contribute to receptor higher order association by changing receptor complex formation and stability. Moreover, inhibition study indicates the focal adhesion kinase (FAK) may be involved in ERBB2 signaling induced GRB7
phosphorylation, suggesting the receptor cluster association with GRB7 results in the engagement of diverse signaling pathways.

4.2 Background

GRB7 was originally identified as a downstream adaptor for EGFR by screening receptor binding targets (Margolis, Silvennoinen et al. 1992). Its aberrant overexpression has been found in numerous cancers, including breast cancer, gastric cancer, esophageal carcinoma and ovarian carcinogenesis (Stein, Wu et al. 1994, Kishi, Sasaki et al. 1997, Tanaka, Mori et al. 1997, Tanaka, Mori et al. 1998, Wang, Chan et al. 2010). The GRB7 family protein includes GRB7, GRB10 and GRB14, all of which contain an N-terminal proline-rich domain interacting with SH3-domain containing proteins, followed by an RA domain which binds to Ras superfamily effector, the PH domain binding to phosphoinositides, a BPS region (Between PH and SH2 ) interacting with IR/IGFR, and a C-terminal SH2 domain recruited to phosphotyrosines of growth factor receptors and intracellular signaling proteins (Shen and Guan 2004) (Figure 4.1). The central segment consisting of the RA and PH domains is also referred as the GM region because it is critical for promoting cell migration during embryogenesis in *C. elegans* (Manser, Roonprapunt et al. 1997). In order to induce cell migration signaling, GRB7 needs to be phosphorylated by FAK (Focal Adhesion Kinase) in a PI3 kinase-dependent manner (Figure 4.2). This phosphorylation event could disrupt the dimeric conformation of GRB7, providing access to the monomeric RA-PH for interaction with other intracellular proteins (Siamakpour-Reihani, Argiros et al. 2009).
GRB7 was identified as one of the genes conferring estrogen independence and tamoxifen resistance to late stage breast cancer cells, which provides high significance for predicting tumor progression in breast cancer patients (van Agthoven, Sieuwerts et al. 2009, van Agthoven, Veldscholte et al. 2009, Godinho, Meijer et al. 2011). A GRB7 peptide inhibitor targeting SH2 domain can efficiently inhibit GRB7 association with phosphorylated ERBB receptors, especially with ERBB3 (David N. Krag 2002). This peptide can cooperate with
trastuzumab to inhibit the growth of SKBR3 cells (Pero, Shukla et al. 2007), suggesting the suppression of receptor association with its downstream adaptors can contribute to the inhibition of receptor signaling.

In human ERBB2 positive breast cancer, GRB7 is co-amplified with ERBB2 on chromosome 17q12 (Stein, Wu et al. 1994). So far the only identified interaction mode between GRB7 and ERBB2/ERBB3 is mediated by SH2 domain binding to ERBB2 at phosphorylated Tyr1139 and ERBB3 at Tyr 1180/Tyr1243. (Janes, Lackmann et al. 1997). However, in our study, GRB7 binding to ERBB2 was increased as ERBB2 overexpressed BT474 cells were treated with kinase inhibitor lapatinib. Based on this result, we proposed and evaluated the hypothesis that GRB7 might interact with ERBB2 in a second novel pattern which is independent of SH2 domain and receptor tyrosine phosphorylation. Combined with our demonstration that FAK might phosphorylate GRB7 in an ERBB2 signaling mediated manner, we proposed a preliminary model that GRB7 monomer-dimer equilibrium shifted by its phosphorylation status can cause differences in adaptor recruitment to ERBB2/ERBB3 receptor, leading to diverse signaling outcomes.

4.3 Materials and Methods

Reagents

Antibodies were obtained from Santa Cruz Biotechnology (ERBB3/C17 and GRB7/N20), Upstate Biotechnology (pTyr/4G10 and the pTyr conjugated beads), Epitomics [ERBB2(CB11) and ERBB2-pY1139]. The purification of Trx-NRG fusion protein of the EGF-like domain of NRG1-β1 with thioredoxin has been
described previously. Lapatinib and canertinib were purchased from LC laboratories, FAK inhibitor 14 from Santa Cruz Biotechnology and neutravidin argose resin from Pierce.

**Cloning and mutagenesis**

ERBB2 constructs were expressed in the pFLAG-MYC-CMV-19 expression vector (Sigma) as before. Flag-tag and His-Biotin tag were fused to GRB7-SH2, GRB7-ΔSH2, or GRB7-FL at N-terminal and C-terminal respectively. This series of truncates were inserted to pEGFP-N1 (Clontech) using EcoRI and NotI. Y188/338F and Y480/492F were generated by following the procedure described by the mutagenesis kit from Agilent (#210515). GRB7 in the CMV vector is a gift from Dr. Lyons in NMSU.

**Cell Culture and Transfection**

MCF7 and BT474 cells were maintained in RPMI-1640 [10% (vol/vol) FBS, 5% (vol/vol) CO2]. 10^6 cells were seeded in 6-well plate two days before experiments. As the confluency reaches 60-70%, 1.5μg of plasmids (0.5μg for each of ERBB2, GRB7 mutant, and BirA) in total were transfected into cells.

**ERBB2 Antibody Coupling**

In order to eliminate the unspecific bands caused by immunoprecipitation, we covalently conjugated ERBB2 antibody with CNBr-activated matrices. 0.03g CNBR powder was swelled in cold 1mM HCl for 1h on ice; Wash the resin with distilled water, and then wash with coupling buffer; Immediately transfer it to coupling buffer containing 0.5mg CB11; Rotate overnight at 4°C; Wash away unreacted ligand using coupling buffer; Block unreacted groups with 0.2M
glycine, pH8.0 for 16h at 4C; Wash with coupling buffer and acetate buffer alternatively for 4 times; Equilibrate resin in PBS with 0.5% NaN3. Coupling buffer: 0.1M NaHCO3/0.5M NaCl pH8.3-8.5; Acetate buffer: 0.1M sodium acetate; 0.5M NaCl.

Coimmunoprecipitation and Neutravidin pull down
Indicated cell lines at 60-70% confluency were treated with 300nM lapatinib for 1h or 24h. Cells were lysed in mild lysis buffer (20 mM Tris, 137mM NaCl, 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 5 mM EDTA, 1mM sodium orthovanadate, 1 mM PMSF). Cell debris was removed by centrifugation for 15min at 16,000×g before immunoprecipitation of supernatants at 4°C for overnight, three washes in mild lysis buffer and denaturation in 1×SDS sample buffer containing DTT. For neutravidin pull down, the neutravidin beads were applied for precipitation.

4.4 Results
4.4.1 GRB7 can bind to non-phosphorylated ERBB2 through a novel mechanism independent of the SH2 domain
We created unphosphorylated ERBB2 receptor by treating ERBB2 overexpressed BT474 cells with the kinase inhibitor lapatinib. Binding studies demonstrate Grb7 recruitment to ERBB2 was not disrupted upon drug treatment, implying that a novel second mode of binding may exist (Figure 4.2). To confirm the possibility that GRB7 may bind to ERBB2 in a phophotyrosine and SH2 independent manner, we generated several truncated versions of GRB7 gene as shown in Figure 4.3 A, including GRB7 full length, SH2 domain only and ΔSH2
module. All the GRB7 constructs were fused with an N-terminal Flag tag, and C-terminal Bio-His-EGFP tag. MCF7 cells transiently co-transfected with GRB7 mutants/ERBB2 vector/BirA vector were subjected to 24h of lapatinib treatment followed by neutravidin pull down.

<table>
<thead>
<tr>
<th>Input</th>
<th>-</th>
<th>+</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lapatinib</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERBB2</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PY</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-ERBB2 (PY1139)</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grb7</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.2 BT474 cells were treated with 300nM lapatinib for 24 hours. Cell lysates were subjected to coimmunoprecipitation using ERBB2 C-terminal antibody CB11. After that, ERBB2 interaction with GRB7 and ERBB2 tyrosine phosphorylation are probed.

Figure 4.3. GRB7 binds to ERBB2 in a novel receptor phosphotyrosine independent manner. A. Differently truncated versions of GRB7 with N-terminal Flag-tag and C-terminal Bio/his-tag have been constructed into pEGFP vector. B. MCF7 cells at 60% confluency were co-transfected with pCMV-ERBB2 plasmid, BirA plasmid, and mutated vector of GRB7. One day later, cells were exposed in 300nM lapatinib for 24 hours. Lysates were applied to neutravidin pull down, and then probed for ERBB2, GRB7 and total phosphotyrosine.
In Figure 4.3B, protein levels of ERBB2 and GRB7 truncates were not altered by long-term lapatinib treatment. After pulling down GRB7 truncated mutations, both SH2 and ΔSH2 domains can be recruited to ERBB2 receptor, with much stronger lapatinib sensitivity for the SH2 domain than ΔSH2. Consistently, by probing receptor tyrosine phosphorylation after Grb7 pull down, the sSH2 interaction with the phosphorylated receptor species is suppressed by lapatinib treatment whereas no phosphorylated receptor can be detected to associate with ΔSH2 at all. This indicates that GRB7 binds to ERBB2 in two ways: (1) The canonical receptor phosphotyrosine dependent mode mediated by SH2 domain. (2) A phosphotyrosine independent mode mediated by an unidentified domain that is not SH2.

4.4.2 Focal Adhesion Kinase (FAK) may play a role in the ERBB2 signaling pathway leading to GRB7 phosphorylation

The earlier literature described that GRB7 was a direct substrate of FAK. In our study, we wanted to know whether FAK is also involved in the ERBB2 receptor mediated GRB7 phosphorylation pathway. To this end, we inhibited FAK kinase activity by using FAK inhibitor 14, which is a selective FAK inhibitor that has minimal specificity for other receptor tyrosine kinases including PDGFR, EGFR, and IGF-RI and can prevent FAK autophosphorylation at Tyr397. To determine the proper duration and concentration for FAK inhibitor to be effective in our system, we did some preliminary experiments as shown in Figure 4.4 A. MCF7 cells were transfected with GRB7 plasmid and cell lysates were applied to neutravidin pull down to probe GRB7 protein level. Figure 4.4 A shows that
GRB7 stability became dramatically reduced at inhibitor concentrations above 5μM. However, the effect of 1μM inhibitor is minimal.

In order to keep a relatively constant GRB7 level, but to inhibit the FAK activity sufficiently, we choose to treat the BT474 cells with 1 μM of FAK inhibitor 14 for 21 hours, Followed by analysis of ERBB2 and GRB7 protein levels (Figure 4.4B). In this ERBB2 overexpressed BT474 cell, NRG stimulation can increase ERBB3 phosphorylation, but frequently reduces overall tyrosine phosphorylation of ERBB2 (Zhang, Park et al. 2012). Tyrosine phosphorylation of GRB7 was drastically elevated by ligand. As FAK inhibitor 14 was added, ERBB2 activation was not significantly affected, but the phosphorylation of GRB7 was notably weakened, suggesting that FAK is required in this ERBB2/ERBB3 signaling mediated tyrosine phosphorylation of GRB7. Alternatively, the FAK inhibitor may
interfere with complex formation of GRB7 with ERBBs without disturbing receptor activity.

4.4.3 GRB7 is recruited to ERBB3 only in phosphotyrosine-dependent manner, and this interaction is increased upon ligand stimulation

Figure 4.5. Only phosphorylated ERBB3 is capable of recruiting GRB7. Truncates of GRB7 with N-terminal Flag-tag and C-terminal Bio/his-tag were constructed into pEGFP vector. MCF7 cells at 60% confluency were co-transfected with pFLAG-ERBB2 plasmid, BirA plasmid, and truncated vectors of GRB7. One day later, cells were exposed in 10nM NRG for 24 hours. Lysates were applied to neutravidin pull down, and then probed for ERBB3 and GRB7.

Fiddes RJ has reported that upon ligand stimulation, GRB7 can be also recruited to ERBB3 at major and minor tyrosine sites of 1180 and 1243 in vivo (Fiddes, Campbell et al. 1998). We would like to know whether the mode of GRB7 binding to ERBB3 might be distinct from ERBB2. The GRB7 truncated mutations were individually cotransfected with ERBB2 into MCF7 cells, and we performed a biotin pull down assay of GRB7 followed by probe for ERBB3 after ligand stimulation. As shown in figure 4.5, the interaction of both GRB7-SH2 and GRB7 full length adaptor with ERBB3 was significantly enhanced after ligand-induced
ERBB3 tyrosine phosphorylation. However, in contrast to ERBB2, ERBB3 did not bind to the GRB7 construct lacking the SH2 domain, suggesting that these two receptors have distinct modes of adaptor recruitment.

4.5 Discussion

GRB7 has been identified as a downstream adaptor of ERBB2 leading to cell proliferation signaling. Traditionally, it was thought to interact exclusively with the tail region of tyrosine phosphorylated receptors, including ERBB2 and ERBB3. However, we found that even when receptor tyrosine phosphorylation is inhibited by kinase inhibitor lapatinib, GRB7 binding to unphosphorylated ERBB2 is still retained. Hence, we propose that another domain in GRB7 promotes its binding to unphosphorylated ERBB2. By creating different truncated mutants of GRB7, we have identified a second novel interaction mode for GRB7 recruitment to ERBB2. This interaction is insensitive to the different conformation imposed on the kinase domain by either type I (active state, canertinib) or type II (inactive state, lapatinib) kinase inhibitors (data now shown).

One conceivable question is: Whether these two modes of binding are mutually exclusive or synergistic? A previous study reveals that the additional interaction between Grb14 and IR is mediated by BPS region. This BPS region binds as a pseudosubstrate in the substrate binding pocket of IR kinase domain to inhibit substrate phosphorylation. The role of the SH2 domain is to increase the binding affinity of the BPS region (He, Rose et al. 1998, Stein, Gustafson et al. 2001, Depetris, Hu et al. 2005). Based on this observation, we predict the novel secondary mode of Grb7 binding to ERBB2 might also be synergistically
impacting the canonical SH2 domain dependent binding. Despite the absence of activation loop in ERBB2, one recent study states that BPS deletion construct results in much more efficient ERBB2 phosphorylation, AKT phosphorylation, and cell transformation, compare to the wild type GRB7 (Saito, Kato et al. 2012). As GRB7 is also capable of binding to ERBB3 at phosphotyrosine docking sites, we then compared the recruitment pattern of GRB7 to ERBB2 and ERBB3. The pull down assay suggests that in contrary to the dual interaction mode with ERBB2, GRB7 can only associate with ERBB3 in receptor phosphorylation dependent way through SH2 domain and this association is increased upon ligand stimulation. Together with the finding that GRB7-SH2 domain exists in equilibrium between monomer and dimer (Porter, Wilce et al 2005.), it is possible that the dimerized GBR7 can be recruited to ERBB2/ERBB3 complex through either SH2-dependent or independent ways. In this case, the receptor complex formation can be changed by altering the binding modes of GRB7 to ERBB2/ERBB3 higher order structure. Previous studies reported that in the integrin induced cell adhesion cascade, GRB7 can be directly phosphorylated by activated FAK with the aid of PI3 kinase. We therefore postulate that the tyrosine phosphorylation of GRB7 mediated by ERBB2/ERBB3 signaling may also be carried out by FAK. In figure 4.4, GRB7 phosphotyrosine invoked by ligand stimulation is notably blocked by FAK inhibitors, suggesting the contribution of focal adhesion kinase to ERBB2/ERBB3 signaling mediated GRB7 phosphorylation. Some biophysical studies have shown that phosphorylated GRB7 or SH2 domain can cause dimerization
disruption and thereby affect its interaction with ERBB2 or other proteins (Siamakpour-Reihani, Argiros et al. 2009, Peterson, Benallie et al. 2012). Hence, the phosphorylation of GRB7 by FAK may function as a regulatory switch to shift the recruitment mode of GRB7 to ERBB2.
Chapter 5 Discussion and Significance

The overexpression of oncogenic protein ERBB2, which accounts for 20-30% of female breast cancers, is associated with poor prognosis and enhanced metastatic potential. The ERBB2/ERBB3 heterocomplex constitutes the most mitogenic signaling pair among the ERBB family. The longstanding question has been how ERBB2 converts extracellular stimuli into cancerous signaling and how this process is spatially regulated by itself. My thesis work has uncovered the mechanism of ERBB2/ERBB3 signaling control from the following aspects.

1) We solved the long-term challenging problem of how ligand-binding deficient ERBB2 is phosphorylated in complex with ERBB3 considering that the latter has diminished kinase activity. Using an RNA aptamer capable of disrupting ERBB3 oligomerization, we have shown that ERBB2 and ERBB3 associate to form tetramer or even higher order structures through side-by-side oriented heterodimers to facilitate proxy activation.

2) We found ERBB2 function can be regulated by serine phosphorylation at its carboxyl terminal tail. These serine phosphorylation events regulate ERBB2 tyrosine phosphorylation. We identified p38 as a putative kinase mediating this regulation.

3) As a downstream adaptor co-amplified with ERBB2 and associated with more aberrant condition of breast cancer, GRB7 can be recruited to ERBB2 in two ways: an SH2 and receptor phosphotyrosine dependent manner, which is sensitive to lapatinib inhibition; and an SH2 & phospho-ERBB2 independent manner, which is irresponsible to kinase inhibitors. In contrast, ERBB3 only
utilizes the canonical mode to recruit GRB7, meaning it is strictly dependent on GRB7SH2 and ERBB3 tyrosine phosphorylation. These different binding modes between ERBB receptors or secondary layers of signaling, such as cross-talk to focal adhesion kinase, may add structural support for and functionally modulate receptor higher order complex formation.

**Receptor higher order association creates functional signaling beyond canonical ERBB2/ERBB3 heterodimers**

Our study about the mechanism of A30 action changed the prevailing model of dimerization-induced ERBB2/ERBB3 activation. As a consequence of A30 inhibition during ligand induced ERBB2/ERBB3 signaling, ERBB2 tyrosine phosphorylation and downstream MAPK signaling are significantly suppressed, whereas ERBB3 tyrosine phosphorylation, AKT activation, and ERBB2/ERBB3 heterodimerization, are only slightly inhibited by A30. Hence, the receptor higher order complex beyond canonical dimers provides the structural framework for ERBB2 phosphorylation to occur in proxy. In this large complex, ERBB3 effectively serves as the scaffold to mediate the association between the ERBB2 receptors.

The inhibitory mode of A30 is functionally similar to that of herceptin, which is now believed to disrupt a non-canonical second ERBB2/ERBB3 interface by targeting ERBB2. This secondary interface mediates the formation of receptor higher order structure from isolated heterodimers that do not constitute functional receptor complex. Also, A30 can amplify the inhibition of ERBB2/ERBB3 tyrosine...
phosphorylation by pertuzumab in ERBB2 positive cells. Since pertuzumab disrupts the heterodimerization interface and aptamer binds to another site, this synergistic inhibition pattern by mechanistically different inhibitors suggests that receptor phosphorylation can happen on two distinct interfaces, and ERBB3 may provide the driving force for efficient HER2 phosphorylation.

Hence, higher order complexes do not only exist for ERBB2/ERBB3, they are a basic mechanistic component needed to qualitatively create the ERBB2/ERBB3 signaling as we know it. Furthermore, these secondary interactions are crucial for normal signaling, even at low endogenous receptor levels that have so far been out of reach for the direct biophysical studies that first reported on the existence of higher order clusters.

**Serine phosphorylation at the ERBB2 C-terminal tail of ERBB2 may serves to attenuate ERBB2 phosphorylation**

The above receptor higher order association model in ERBB2 overexpressed cells provides a platform for two ERBB2 receptors to phosphorylate each other mediated by the low amount of ERBB3. The allosteric activation and transphosphorylation which requires the contacts between the kinase domain of one ERBB2 receptor with carboxyl terminal tail of another one raises the possibility of regulation of the receptor kinase activity by alterations at carboxyl terminus.

We found that p38 mediated serine phosphorylation at the ERBB2 carboxyl terminal tail could inhibit receptor tyrosine phosphorylation. As serines were
replaced by alanines, these mutants display a 2-3 fold increase in receptor tyrosine phosphorylation. Fusion of a GFP sized C-terminal Dendra protein appears to enhance the degradation of ERBB2 receptor, suggesting by changing the overall structure and conformation of the tail region, the receptor endocytotic pathway may also be re-routed.

We proposed a possible model to describe the mechanism of observed serine phosphorylation inhibition on receptor tyrosine phosphorylation in ERBB2 overexpressed cells. During the receptor tyrosine phosphorylation process, the tail region of one ERBB2 receptor (A) interacts with the kinase domain of another ERBB2 receptor (B). The phosphorylated serine residues at ERBB2 carboxyl terminal tail can create steric interfere to disrupt the catalysis between kinase domain of receptor (B) and the tyrosine residues on receptor (A), resulting in inhibited receptor tyrosine phosphorylation.

Moreover, alternative models to explain the negative regulatory role may involve the recruitment of phosphatases to the phosphotyrosine sites at the carboxyl tail region of ERBB2 upon serine phosphorylation of the receptor. The phosphatase activation might be directly initiated by serine kinases such that ERBB2 phosphotyrosine is removed by the phosphatase.

can also trigger Bcl2 family protein mediated-apoptosis cascades within mitochondria (Bulavin and Fornace 2004). Another intriguing study indicates that p38 acts as a sensor of reactive oxygen species (ROS) at the starting point of tumorigenesis to protect against oncoproteins which generate large amount of ROS. Neu is such a protein implicated with increased transformation of p38α-deficient fibroblasts (Dolado, Swat et al. 2007).

In cancer patients, the role of p38 to suppress tumor progression is compromised. A systematic genome screening study suggests that components of the p38 pathway as well as p38 isoforms were mutated and act as ‘driver’ mutations leading to the development of human cancers (Greenman, Stephens et al. 2007).

In lung cancer, p38α expression is found to be frequently suppressed (Ventura, Tenbaum et al. 2007). Accordingly, the activator of p38 -- M KK4 is downregulated in several types of metastatic tumors (Robinson, Hickson et al. 2003). Hence, the inhibitory effect of p38 on oncogenesis makes it an attractive target in cancer prevention and treatment. This could be achieved by activation of p38 cascade or removal of its suppressors, such as p38 MAPK phosphatase Wip1. Pharmacologic activation of p38 MAPK could restrain tumor development by keeping ERBB2 tyrosine phosphorylation at a relatively lower level. The p38 phosphatase Wip1 is overexpressed in certain human cancers and siRNA based-depletion of Wip1 phosphatase has already been shown to cause Wip1-overexpressing cancer cells to undergo apoptosis (Hirasawa, Saito-Ohara et al. 2003). In mouse model with deletion of the gene encoding Wip1, mammary tumorigenesis was inhibited for mice with MMTV promoter-driven ERBB2 gene
(c-neu) through the activation of p16/p19 pathway (Bulavin, Phillips et al. 2004). However, one potential problem is that chronically activated p38 may also generate chronic stress to the cells, which might elicit protection mechanism for the cells to resist the continuous activation of p38.

In summary, activation of p38 MAPK may serve as a new therapeutic approach for inhibiting oncogenic pathways in human cancers, and thereby is a promising target for developing novel anti-cancer drugs. However, more intensive investigations are required to dissect the role of p38 MAPK signaling in regulating tumor progression through ERBB2 pathway.

The multi-domain binding mode of GRB7 towards ERBB2/ERBB3 might diversify the signaling outcomes emanating from higher order receptor complexes

Previous literature showed that GRB7 can interact with ERBB2 or ERBB3 individually in a receptor tyrosine phosphorylation-dependent manner. In this study, we provide strong evidence to demonstrate that GRB7 is recruited to ERBB2 through two modes: 1) phosphotyrosine dependent binding mediated by SH2 domain and 2) Phosphotyrosine independent binding mediated by an unidentified but probably region. The former binding mode is sensitive to lapatinib inhibition whereas the latter is not (Figure 4.3). However, in constrast to ERBB2, ERBB3 can only associate with GRB7 when the receptor is tyrosine phosphorylated.
Which regions on ERBB2 and GRB7 are involved in mediating the secondary association? It is possible that the receptor kinase domain is the binding target for the GRB7-BPS region. Previous studies have shown that an additional interaction between GRB7/GRB10/14 and Insulin Receptor (IR) is mediated by the BPS domain of the adaptors and the kinase domain of IR (Kasus-Jacobi, Bereziat et al. 2000, Desbuquois, Carré et al. 2013). More importantly, crystal structures reveal that the BPS region binds as a pseudosubstrate in the substrate binding pocket of the IR kinase domain to inhibit substrate peptide phosphorylation (Depetris, Hu et al. 2005).

What is the nature and biological significance of the dual binding modes? These differences in the mode of association highlight that the changes in adapter recruitment are likely to be a result or in the reverse aid in the alterations of receptor higher order structure. The reported ability of GRB7 to alternate between a monomeric and dimeric state further adds diversity to this recruitment platform (Peterson, Benallie et al. 2012). It is quite possible that either dimeric or monomeric GRB7 is recruited to ERBB2/ERBB3, such that receptor higher order structure can be stabilized or destabilized by shifting the equilibrium of alternative multi-domain binding modes of GRB7 (Figure 5.1). This high level complexity of receptor-adaptor association eventually could contribute to the extreme diversity of signaling outcomes that are experimentally observed. Loss of select interaction modes of GRB7 can dramatically alter signaling outcomes. GRB7v is a natural splicing variant of GRB7 whose C-terminal SH2 domain is replaced by a small hydrophobic segment. Evaluation of signaling outcomes of GRB7 and
GRB7v in ovarian cancer shows that GRB7v contributes primarily to cell proliferation and cell growth through the ERK1/2 pathway, whereas the unregulation of non-truncated GRB7 resulted in cell migration, invasion and proliferation by simultaneously activating all of ERK, AKT, and c-Jun kinase signaling (Wang, Chan et al. 2010). Hence, the dual binding modes may confer signaling to a broader spectrum of downstream signaling cascades.

In our studies, the GRB7 tyrosine phosphorylation was sensitive to FAK kinase inhibitor, as shown in Figure 4.4. This GRB7 phosphorylation may confer another layer of regulation to signaling through the disruption of GRB7 dimerization.
(Peterson, Benallie, 2012). A major open question is the nature of FAK involvement since some studies suggest activated ERBB2 receptors and FAK pathway can integrate with each other by using GRB7 as a signaling hub (De Pradip, Dey et al. 2013).

We propose a model for the ligand-induced ERBB2/ERBB3 signaling to mediate GRB7 tyrosine phosphorylation by FAK. In BT474 cells, ligand stimulation induces ERBB2/ERBB3 receptor complex formation, leading to the activation of FAK through an unidentified mechanism. The activated FAK can then phosphorylate GRB7 and disrupt the dimerization. However, the autophosphorylation of ERBB2 under ligand-independent condition is not sufficient to induce FAK activation and GRB7 tyrosine phosphorylation.

Recently, a novel GRB7 inhibitor G7-18NATE binding specifically to the SH2 domain and was developed to block GRB7 association with RTK. The co-
treatment of G7-18NATE with Herceptin in multiple ERBB2 positive breast cancer cell lines can result in cooperative cell growth inhibition (Pero, Shukla et al. 2007). My data provide a model of signaling that integrates higher order structures into ERBB2/ERBB3 signaling, and in a related manner, signaling by overexpressed ERBB2. It is therefore likely that a modulation of these signaling platforms can also influence the mode of GRB7 interaction, and therefore they way in which the crosstalk occurs between ERBB2/ERBB3 and focal adhesion or migration control.
References


