NOTE TO USERS

IN VIVO STRUCTURE-FUNCTION STUDIES OF THE ERBB2 RECEPTOR TYROSINE KINASE

By

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Abstract

The ErbB2 receptor tyrosine kinase plays important roles in both mammalian development and in oncogenesis. Signal transduction mediated by the receptor is dependent on the catalytic activity of its tyrosine kinase domain and the phosphorylation of discrete tyrosine residues within the receptor. To perform structure-function studies in vivo and to evaluate the function of tyrosine phosphorylation, an allelic series of *erbB2* cDNA knock-in animals were generated such that the *erbB2* cDNA was under the transcriptional control of the endogenous promoter. Mice homozygous for the kinase-dead *erbB2* allele died at midgestation and displayed the same spectrum of embryonic defects observed in *erbB2* deficient animals. In contrast, expression of ErbB2 receptors harboring a single tyrosine site mutation in the carboxy-terminus of the receptor resulted in viable animals. However, expression of ErbB2 receptors de-coupled from the Shc signaling pathway (Y1226/7F) lead to subtle defects in the development of cutaneous sensory nerves.

The cDNA knock-in alleles were inadvertently hypomorphic expressing a minimum threshold level of ErbB2 (~10% of wild type) required for survival. Indeed, a further 2-fold reduction in ErbB2 levels in hemizygous knock-in animals resulted in perinatal lethality due to defective innervation of the diaphragm leading to acute respiratory distress. Interestingly, this hypomorphic phenotype was genetically rescued when the ErbB2-Y1028F mutant receptor was expressed in animals using a comparable

knock-in strategy. Subsequent molecular analyses revealed that the Y1028F allele expressed higher levels of ErbB2, due to the decreased turnover rate of the receptor following removal of Y1028. This downregulation was independent of c-Cbl association and ubiquitylation of the receptor. Instead, ErbB2-Y1144 and Y1226/7 can associate with c-Cbl but the receptor remains refractory to c-Cbl mediated ubiquitylation. Additionally, ErbB2 is monoubiquitylated through an unidentified mechanism mediated by the Y1253 phosphorylation site. Taken together, these results provide important novel insight into the function and regulation of the ErbB2 receptor.

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I have experienced an important learning and career building stage in my life as a member of the Muller Lab where I have endured the many ups and downs in becoming a respectable scientist. For this, I must thank Bill first for giving me the opportunity to be a part of his lab and then providing me with the supervision yet also enough freedom to develop scientifically on my own. Ironically, I think my most valued experience and my greatest contribution was for my part in rebuilding the Muller Lab after moving to McGill. I am also indebted to Pete, Rod, and David for their mentorship and friendship in helping me establish a solid foundation from which I owe much of my successes to. Thanks also to Juliet, Roger, Andre, and Michael for being a part of my (current or past) supervisory committee and to Pat Hayward (graduate coordinator) for keeping things on track. Throughout this long road I have crossed paths with many people, many of whom have had a positive influential role in my life both academically and personally. Special appreciation goes to Eran, Steve, Bart, Patrick, and Neera during my time at McMaster and to Lina, Dongmei and Josee in Montreal. Thank you for your true and unconditional friendship. And finally, I must acknowledge and thank my parents for their trust and support in allowing me to make my decisions to get to where I am today.

Contribution by Others

CHAPTER 3

The targeting constructs and animals were generated by Rod Hardy (McMaster).

Construction of the ErbB2 kinase-dead mutant cDNA and the initial in vitro testing of this mutant were performed by Sarah Hardy (McMaster).

In situ analyses and the data presented in Figure 4 were produced by Michael Laing (McMaster).

CHAPTER 4

The knock-in animals were generated by Rod Hardy.

Figure 3 was contributed by Michael. Laing.

CHAPTER 5

The ErbB2 cDNA mutants were generated previously by David Dankort (McMaster).

They Y1117F mutant was constructed by Harold Kim (McMaster).

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List of Abbreviations

AChR	Acetylcholine Receptor
cDNA	Complementary DNA
CSF-1	Colony Stimulating Factor-1
DEPC	Diethylene Pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine-tetra-acetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ENU	Ethylnitrosourea
Erk	Extracellular Regulated Kinase
ES	Embryonic Stem
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
Grb	Growth Factor Receptor Bound Protein
H & E	Hematoxylin and Eosin
HGF	Heregulin
HRP	Horseradish Peroxidase
IB	Immunoblot
IP	Immunoprecipitation
KD	Kinase Dead or Kinase Deficient
KI	Knock-in
KO	Knock-out
mAb	Monoclonal Antibodies
MAPK	Mitogen Activated Protein Kinase
MEK	MAPK/ERK Kinase
MMTV	Mouse Mammary Tumor Virus
MVB	Multivesicular Body
NDF	Neu Differentiation Factor
Neo	Neomycin

NF	Neurofilament
NRG	Neuregulin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGFR	Platelet Derived Growth Factor Receptor
PGK	Phosphoglycerate Kinase
PI3'K	Phosphatidyl Inositol 3' Kinase
PLCγ1	Phospholipase Cγ1
PMSF	Phenylmethylsulfonyl fluoride
PNS	Peripheral Nervous System
PTB	Protein Tyrosine Binding
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
PVDF	Polyvinylidene Difluoride
Ras-GAP	Ras GTPase-Activating Protein
RNA	Ribonucleic Acid
RNase	Ribonuclease
RTK	Receptor Tyrosine Kinase
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulfate
SH2	Src Homology 2
Shc	SH2 and Collagen Homology Proteins
SOS	Son of Sevenless
TBS	Tris Buffered Saline
TGFα	Transforming Growth Factorα
TKB	Tyrosine Kinase Binding
TM	Transmembrane
Ub	Ubiquitin
UIM	Ubiquitin-Interacting Motif
WT	Wild Type

Chapter 1

Introduction

The transfer of information from extracellular cues into the cell is primarily mediated by Receptor Tyrosine Kinases (RTK) spanning the plasma membrane. One important group of receptors is the Epidermal Growth Factor Receptor (EGFR) or ErbB family, which along with their downstream signaling pathways play prominent roles in the control of cellular growth, differentiation, migration and survival in a variety of physiological processes. Consequently, aberrant activities of these receptors have been implicated in a number of human cancers including breast, ovarian, and prostate. While understanding the molecular pathology of the ErbB family involved in cancer is of obvious clinical importance, it is equally relevant and informative to realize the normal activities of these receptors. The ErbB receptors also are involved in several developmental roles as demonstrated by the embryonic lethality and tissue defects associated with interrupting their expression in gene knock-out mouse models. By exploiting the developmental roles of the ErbB group, this provides an alternative approach to understanding the molecular details of this receptor family through structure-function studies. This valuable information may subsequently be applied in clinical therapeutic strategies against cancer.

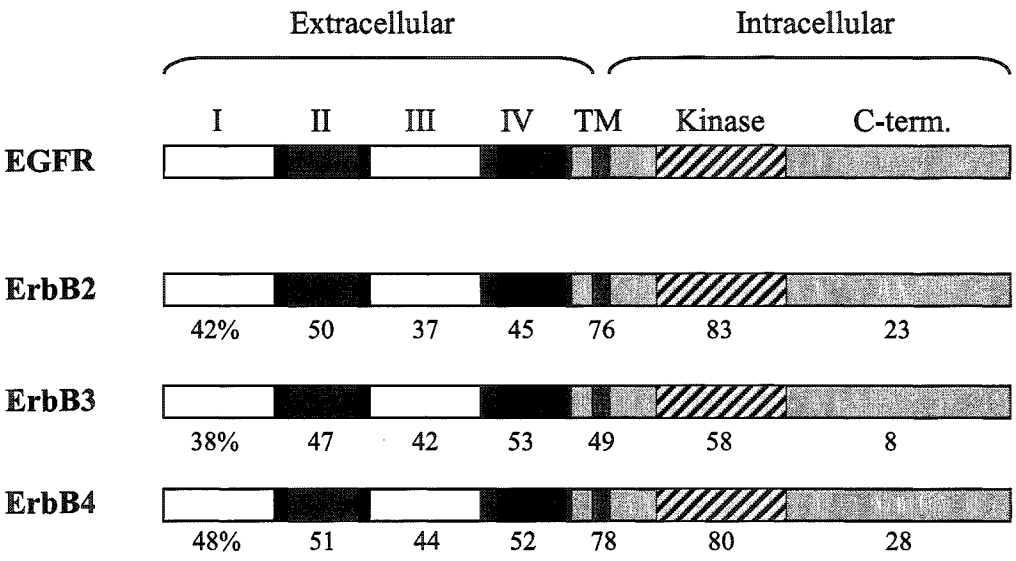
1.1 The EGFR Family of Receptor Tyrosine Kinases

The Epidermal Growth Factor Receptor is the founding member of what is now called the EGFR or ErbB family of receptor tyrosine kinases (RTK) (Carpenter et al., 1978). In addition to the EGFR (ErbB1, HER1 for Human EGF Receptor 1), this family now consists of three additional members: ErbB2 (Neu in rat; HER2 in human), ErbB3 (HER3), and ErbB4 (HER4) (Bargmann et al., 1986b; Coussens et al., 1985; Downward et al., 1984; Kraus et al., 1989; Plowman et al., 1993; Plowman et al., 1990; Yamamoto et al., 1986). Together, they represent the Type I class of receptors, sharing similar structural characteristics including an extracellular ligand binding domain, a single pass hydrophobic transmembrane domain, a highly conserved catalytic protein tyrosine kinase (TK) core followed by a carboxyl terminal stretch containing several critical tyrosine residues (Ullrich and Schlessinger, 1990). Within the family, there is an overall 40-50% amino acid identity with the greatest homology in the tyrosine kinase domain (58-83%)(Earp et al., 1995), suggesting that it is essential for these receptor's activity, whereas the C-terminal region is the most divergent (8-23%) (Peles and Yarden, 1993) (Figure 1.1). Overall, the structure is evolutionarily conserved from invertebrates, namely the *Caenorhabditis elegans* Let-23 receptor and the *Drosophila* DER receptor orthologs of the EGFR.

The extracellular region of the ErbB family of RTKs is comprised of four distinct subdomains (Figure 1.1): two homologous ligand binding domains (I and III) and two cysteine-rich domains (II and IV). One of the functions of subdomains II and IV is to maintain an autoinhibited configuration through intramolecular contacts, thereby forcing

Figure 1.1 Structural organization of the EGFR/ErbB family of RTKs

Schematic diagram (adapted from Brennan et al., 2000) of the four ErbB RTKs showing the common functional domains and the degree (%) of amino acid similarities relative to the EGFR. The extracellular domain is divided into four subdomains (I-IV). I and III form the ligand binding domain whereas II and IV are the cysteine-rich regions and contribute to the dimerization interface. There is a single transmembrane (TM) domain, followed by a highly conserved catalytic tyrosine kinase domain (Kinase). The carboxy-terminus (C-term) is the most divergent domain, which harbors several potential tyrosine autophosphorylation sites.


Extracellular
Intracellular
I
II
III
IV
TM
Kinase
C-term.
EGFR
ErbB2
42%
50
37
45
76
83
23
ErbB3
38%
47
42
53
49
58
8
ErbB4
48%
51
44
52
78
80
28

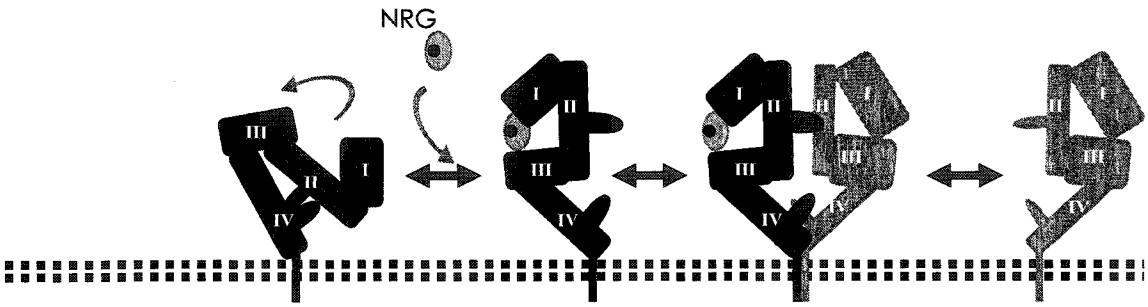
subdomains I and III into a relative orientation that prevents high-affinity binding of a ligand (Figure 1.2) (Cho and Leahy, 2002; Ferguson et al., 2003; Lax et al., 1989). Once a ligand binds to subdomains I and III, this alters the configuration of the extracellular region and exposes a dimerization arm located in subdomain II, thus allowing it to make intermolecular links with an adjacent receptor and driving dimerization (Ferguson et al., 2003).

The tyrosine kinase domain is responsible for the transfer of a phosphate group from an ATP molecule to a tyrosine residue on a protein substrate. As mentioned above, the most highly conserved region within all ErbB family members, as is the case with most other RTKs in general, is the tyrosine kinase domain. One noteworthy exception however is the ErbB3 protein. This receptor shares the least sequence identity with the other ErbB receptors, including residues that are critically conserved throughout all protein kinases, thus rendering it catalytically inactive (Guy et al., 1994b). Within the kinase domain is a lysine residue that functions essentially as the ATP binding site and is critically required for the enzymatic activity. Replacement of this consensus lysine residue in any RTK invariably abolishes the ability of the receptor's kinase domain to phosphorylate its substrates (Chen et al., 1987; Chou et al., 1987; Honegger et al., 1987; McClain et al., 1987; Qian et al., 1994a; Russell et al., 1987; Williams, 1989).

The carboxy-terminal tail sequences are among the most divergent between ErbB receptors (Peles and Yarden, 1993). However, not to be limited by this, it is likely that the heterogeneity predominantly accounts for the differences or specificity of receptor tyrosine kinase signaling (Di Fiore et al., 1990). Within the variability of the carboxy-

Figure 1.2 Model of ErbB receptor activation and dimerization

At left, an inactive, tethered ErbB3 monomer showing the relative orientation and intramolecular interactions amongst the four subdomains. These are based on the recently resolved crystal structures of the receptors' ectodomain. Upon ligand (neuregulin, NRG) binding (subdomains I and III), this promotes the extended configuration and exposes a dimerization interface (subdomain II and IV). On the right is an ErbB2 receptor that is constitutively in the extended configuration and does not require ligand binding. Preferential heterodimerization between ErbB3 and ErbB2 results in a potent signaling unit. For simplicity, only the extracellular domain is shown. (adapted from Burgess et al., 2003)


NRG
I
II
III
IV
ErbB3
monomer
ErbB3/
NRG-bound
ErbB3/ErbB2
heterodimer
ErbB2
monomer

terminus lies several conserved tyrosine residues, as well as the few amino acids just upstream and/or downstream of these tyrosine residues. Sequence alignment comparing the carboxy-terminus shows the highest level of conservation between the EGFR and ErbB2 receptors, particularly with the several tyrosine autophosphorylation sites that have previously been mapped in the carboxy-terminus of the EGFR and ErbB2 (Dankort et al., 1997; Hazan et al., 1990; Margolis et al., 1989). It is these phosphorylated residues that are recognized and bound by specific intracellular proteins, thereby initiating a highly coordinated signal cascade.

1.2 The ErbB2/Neu Receptor Tyrosine Kinase

1.2.1 The Neu Oncogene

Initially, the *neu* oncogene (or rat *erbB2*) was identified in transfection studies using DNA isolated from the chemical carcinogen, ethylnitrosourea (ENU), induced rat neuroblastomas (Shih et al., 1981). The result was the isolation of a 185kDa phosphoprotein that was capable of morphologically transforming NIH3T3 fibroblast cells (Padhy et al., 1982). A comparison of the p185 protein revealed that it had a high degree of homology to the retrovirally transduced v-erbB/EGF receptor (Downward et al., 1984; Schechter et al., 1984). A human cDNA encoding the *c-neu/c-erbB2* homolog was subsequently cloned by screening cDNA libraries using the viral *v-erbB* sequences as a probe under low stringency hybridization conditions (Coussens et al., 1985). This gene represents the human ortholog of the rat *neu*, and is termed *erbB2* or *her2*. Subsequent isolation of the rat cDNA (Bargmann et al., 1986b) encoding the *neu* (neuroblastoma)

oncogene and sequence comparison with the wild type form of *neu* revealed a single amino acid point mutation located within the transmembrane region of the oncogenic receptor (Bargmann et al., 1986a). This substitution resulted in the conversion of a valine residue (amino acid 664) to a glutamic acid (V664E mutation). The consequence of this single mutation is significant since it confers upon the receptor a ligand-independent and constitutive catalytic activity (Bargmann and Weinberg, 1988; Stern et al., 1988; Weiner et al., 1989b). Structurally, the V664E mutation promotes Neu receptor oligomerization evidently by stabilizing the two α-helical transmembrane domains through hydrogen bond formation between the receptor dimer (Sternberg and Gullick, 1989; Sternberg and Gullick, 1990; Weiner et al., 1989b). This phenomenon is not unique to the ErbB2/Neu receptor as activating point mutations in the CSF-1 (c-Fms) receptor also induces stabilization of a conformation that mimics a ligand-triggered activated CSF-1 receptor (Roussel et al., 1988; Woolford et al., 1988).

Alternatively, dimerization also may be promoted through intermolecular disulfide bond formation between the cysteine-rich regions in the extracellular domain of the ErbB receptors. It is thought that this occurs through the addition or removal of a cysteine residue resulting in an imbalance or unpaired cysteine residue that may then form the intermolecular link, thereby promoting receptor dimerization. This is based on the identification of spontaneous activating mutations (deletions, point mutations, or insertion mutations) in the Neu receptor isolated from mammary tumors of transgenic mice overexpressing the *neu* proto-oncogene (Chan et al., 1999; Siegel et al., 1994).

1.2.2 ErbB2 and Human Breast Cancer

Elevated expression of the ErbB receptors has been observed in clinical studies of a significant number of sporadic breast cancers. Indeed, overexpression of EGFR in mammary carcinomas inversely correlates with patient outcome (Harris et al., 1989; Sainsbury et al., 1987). Additionally, during the initial cloning experiments to isolate the *erbB3* gene, this was found to be overexpressed in several mammary cancer cell lines (Kraus et al., 1989). Furthermore, a limited number of studies relating the ErbB3 receptor with human breast cancer have concluded that approximately 22% of cases involved elevated levels of ErbB3 (Gasparini et al., 1994; Lemoine et al., 1992; Quinn et al., 1994) however there was no evidence that this was the result of gene amplification (Kraus et al., 1989; Lemoine et al., 1992). Other studies suggest that ErbB3 may be a culprit along with ErbB2 in breast cancer by relating the coincidental elevated co-expression of ErbB2 and ErbB3 in mammary tumors (Siegel et al., 1999) or the requirement for both ErbB2 and ErbB3 to drive breast tumor cell proliferation (Holbro et al., 2003). The role of ErbB4 in mammary tumorigenesis however can only be inferred based on data showing the increased detection of ErbB4 in breast cancer cell lines (Plowman et al., 1993).

Although the relationship between expression of EGFR, ErbB3 and ErbB4 with the induction mammary carcinoma has been documented, it is perhaps not as compelling and well characterized as for the ErbB2 receptor. To this end, a number of clinical studies have estimated that amplification and overexpression of the *erbB2* proto-oncogene is involved in about 20-30% of human breast cancer (Press et al., 1993; Slamon

et al., 1987; Slamon et al., 1989; Venter et al., 1987; Zeillinger et al., 1989). The consequences of this has been correlated with a poor clinical prognosis for the patient with increased chance of relapse and death (Andrulis et al., 1998; Gullick et al., 1991; Paterson et al., 1991; Slamon et al., 1987; Slamon et al., 1989).

1.2.3 ErbB2 Mutations in Cancer

Consistent with other receptors, activating mutations seem to only play a minor role in ErbB2-related human cancers. Indeed, it should be noted however that despite the identification of the V664E activating mutation in the sequence of the rat Neu transmembrane region, a comparable mutation has not been found in human ErbB2 (Lemoine et al., 1990; Slamon et al., 1989). Thus, it appears that expression of *erbB2* to elevated levels either through gene amplification or deregulated expression of wild type *erbB2* may result in aberrant growth. On this note, an alternatively spliced isoform of ErbB2 was detected in human breast tumor samples and this mutant receptor displays elevated catalytic activity in vitro (Kwong and Hung, 1998; Siegel et al., 1999). The alternate splicing results in an ErbB2 receptor that is strikingly similar to the activating deletion mutations identified in Neu-induced murine tumors, which have a higher propensity to dimerize (Siegel and Muller, 1996; Siegel et al., 1999). Indeed, cell transformation assays expressing the alternatively spliced ErbB2 isoform resulted in morphological transformation of Rat1 cells (Siegel et al., 1999). The exact role and function of the alternatively spliced ErbB2 receptor in the normal mammary gland and/or in mammary oncogenesis is not clear at this point.

1.3 ErbB Family RTKs in Development

The first evidence for the essential roles of ErbB receptors in various aspects of mammalian development were revealed by gene targeting studies in animals. The defects associated with disruption of the genes encoding for *neuregulin-1 (NRG-1), erbB2, erbB3* and *erbB4* suggested that Neuregulin-1/ErbB signaling are involved in both cardiac and nervous system development.

1.3.1 Development of the Heart

NRG-1, *erbB2* and *erbB4* deficient animals share a similar midgestation (E10.5) lethality due to malformation of the myocardial trabecules of the ventricle (Britsch et al., 1998; Erickson et al., 1997; Gassmann et al., 1995; Kramer et al., 1996; Lee et al., 1995; Liu et al., 1998; Meyer and Birchmeier, 1995). During heart development, NRG-1 is expressed in the endocardium and acts in a paracrine manner on the co-receptors ErbB2 and ErbB4, which are expressed in the adjacent myocardium (Gassmann et al., 1995; Meyer and Birchmeier, 1995). This signaling module is required for the formation of ventricular trabecules, the finger-like projections of the myocardium that are critical for efficient circulation of the blood. Additional evidence demonstrating the essential function of ErbB2 in the heart is given by two subsequent studies where transgenic expression of an erbB2 cDNA in a myocardium-specific manner rescued the cardiac defects (Morris et al., 1999; Woldeyesus et al., 1999). Strikingly, mutation of ShcA, an adapter protein that functions immediately downstream of ErbB receptors, also results in a similar cardiac phenotype (Lai and Pawson, 2000). It is poorly understood at this time

the molecular and cellular mechanisms within the cardiomyocytes that are responsible for the process of trabeculation. However, cell and embryo culture experiments examining the response to NRG-1 stimulation have produced conflicting results. In one study, NRG-1 promoted the survival and proliferation of cardiomyocytes and induced hypertrophic growth (Zhao et al., 1998). Other studies did not report any proliferative responses in cardiomyocytes unless the cells were co-stimulated with another growth factor such as Insulin-like growth factor (Ford et al., 1999; Hertig et al., 1999). The nature of the discrepancies appears to lie in the influence of culture conditions and/or sensitivities to growth factor combinations based on the developmental stage of the cells.

Interestingly, ErbB2 and ErbB4 also may have important roles in the maintenance and functioning of the adult heart. This first came to attention when the anti-ErbB2 breast cancer drug, Trastuzumab (Herceptin™, Genentech) was used in clinical trials and it became apparent that a significant number of patients started to experience cardiac dysfunction (Seidman et al., 2002; Slamon et al., 2001). These unfortunate side effects of Trastuzumab suggested that the attenuation of ErbB2 signaling in the heart might lead to cardiomyopathies. This conclusion was reiterated in a mouse model where conditional mutagenesis of *erbB2* in ventricular cardiomyocytes resulted in adult animals developing severe dilated cardiomyopathy with compromised contractile function (Crone et al., 2002; Ozcelik et al., 2002).

The other NRG-1 responsive receptor, ErbB3, does not appear to have a role in trabeculation but is important in the formation of the atrioventricular valve separating the atrium and ventricle. ErbB3 is expressed in the mesenchyme of the endocardial cushion,

which is the primordial atrioventricular valve. Consistent with this, in *erbB3* deficient animals trabeculation in the heart is normal whereas the atrioventricular valves are rudimentary and thinned, leading to death in utero at E13.5 (Erickson et al., 1997; Riethmacher et al., 1997).

1.3.2 The Peripheral Nervous System

The peripheral nervous system, namely the sensory and dorsal root ganglion, is predominantly formed from neural crest derived cells, which migrate away from the dorsal neural tube along defined pathways to their target sites (Le Douarin, 1986) and are dependent on molecular cues detected during migration. *NRG-1* and *erbB2* deficient mutant animals, which die at E10.5 due to cardiac defects, as well as *erbB3* deficient animals, also display defects in the peripheral nervous system (Britsch et al., 1998; Erickson et al., 1997; Gassmann et al., 1995; Kramer et al., 1996; Lee et al., 1995; Liu et al., 1998; Meyer and Birchmeier, 1995). In contrast, embryos lacking *erbB4* expression have intact sensory ganglia but the existing axons mis-innervate the hindbrain (Gassmann et al., 1995). ErbB3 is normally expressed at relatively high levels in neural crest cells. In the absence of NRG-1/ErbB signaling, the neural crest derived cranial sensory ganglia and its corresponding axonal connections to the hindbrain are lost and the sympathetic ganglion chain in the mutant embryos are hypoplastic (Britsch et al., 1998; Erickson et al., 1997; Kramer et al., 1996; Meyer and Birchmeier, 1995). The poorly developed primary sympathetic ganglion chain is due to the inability of neural crest cells to migrate to the anlage of the primary chain.

Schwann cell differentiation is specifically controlled by Neuregulins, the essential neuron derived factors that regulate Schwann cell number (Dong et al., 1995). In this case the ErbB2/ErbB3 heterodimer is the active receptor expressed in Schwann cells that respond to the neuron derived NRG (Levi et al., 1995). In fact, NRGs are the only factors capable of supporting the survival of Schwann cell precursors in culture. Owing to the dependency of these myelin-forming cells on the NRG/ErbB signaling, Schwann cell development is compromised in *erbB2* deficient animals, and at later developmental stages they were completely absent in the peripheral nerves (Morris et al., 1999; Woldeyesus et al., 1999). Consequently, peripheral nerves are hypomyelinated, suggesting that the NRG/ErbB signal is responsible for determining the thickness of myelin sheath (Garratt et al., 2000a; Garratt et al., 2000b). Thus, the incomplete myelination is associated with motoneuron neuropathy observed in *erbB2* and *erbB3* mutant animals.

1.4 Receptor Activation

1.4.1 ErbB Ligands

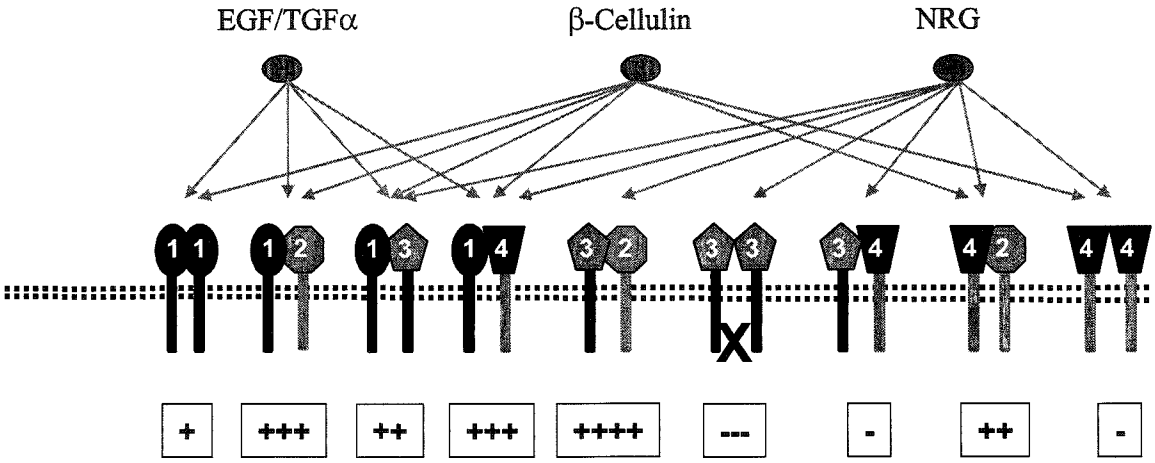
Typically, ErbB receptors are activated by a number of ligands, adding to the potential and diversity of ErbB signaling responses (Figure 1.3) (Tzahar and Yarden, 1998). Each ligand, now commonly referred to as EGF-like peptide, has an EGF-like domain that is sufficient to confer unique specificity for its receptor substrate and they have the ability to modulate the catalytic activity of the receptor. Most of these ligands act over short distances as autocrine or paracrine factors and their availability or

expression pattern in a tissue-specific or developmental stage-specific pattern offer a level of control over their signaling potential. The EGF-like peptides are divided into three groups based on their specificity: epidermal growth factor (EGF), amphiregulin (AR) and transforming growth factor-α (TGF-α) specifically activate EGFR (Normanno et al., 1994); betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR) exhibit dual specificity for EGFR and ErbB4 (Riese and Stern, 1998); and the neuregulins (NRG) bind to both ErbB3 and/or ErbB4. NRG-1 and NRG-2 both can bind to ErbB3 and ErbB4 (Carraway et al., 1997; Chang et al., 1997; Riese et al., 1995) whereas NRG-3 and NRG-4 are specific for ErbB4 and not ErbB3 (Harari et al., 1999; Zhang et al., 1997). It should be noted that alternate splice forms exist for the NRG-1 ligands, and their names reflect the tissue in which they were first discovered. These include Neu differentiation factor (NDF) (Peles et al., 1992a; Wen et al., 1992), heregulin (HRG) (Holmes et al., 1992), acetylcholine receptor-inducing activity (ARIA) (Falls et al., 1993), and glial growth factor (GGF) (Marchionni et al., 1993).

Despite several independent efforts, no direct soluble ligand for ErbB2 has been identified to date and it remains an orphan receptor. In fact, recent structural analyses of the ectodomain of ErbB2 suggest that it may not require ligand binding and remains in a conformation that is conducive to oligomerization (see below). However, there have been reports suggesting that a member of the Mucin family known as Muc4/sialomucin may act as an unconventional intramembrane ligand for ErbB2 (Carraway et al., 1999). Interestingly, it contains EGF-like domains that are similar in sequence to the EGF-like domains in the NRG ligands (Sheng et al., 1992) and is capable of activating ErbB2

Figure 1.3 The ErbB signaling network

Multiplicity and diversity in the ErbB signaling network is depicted schematically (Alroy and Yarden, 1997). Various ligands (only 3 examples are shown) have specificity for distinct receptors to elicit different molecular responses. The four receptors are numbered accordingly: (1) EGFR/ErbB1; (2) ErbB2; (3) ErbB3; (4) ErbB4. Nine receptor homodimerization and heterodimerization combinations are shown, each pair leading to the formation of unique sets of intracellular signaling complexes and signal transduction cascade. The relative strength of the biological response is indicated by (+) for a strong response and (-) for a weaker or no effect. ErbB3 homodimers may form, albeit very weak, and are likely to be signaling inept since the ErbB3 kinase domain is naturally defective.


EGF/TGFα
β-Cellulin
NRG
1 1
1 2
1 3
1 4
3 2
3 3
3 4
4 2
4 4
X
+
+++
++
+++
++++

-
++
-

leading to limited ErbB2 phosphorylation as well as inducing the translocation of ErbB2 from the basolateral surface to the apical surface in polarized epithelial cells (Jepson et al., 2002; Ramsauer et al., 2003). While the exact role and function of this Muc4/ErbB2 interaction is still very much uncharacterized, it will become more interesting as further studies continue to define its role in normal ErbB2 activity.

1.4.2 Receptor Oligomerization

The significance of the deletion and point mutations or stoichiometric overexpression of ErbB receptors is given by the fact that they enhance receptor oligomerization, which is essential for their activation (Siegel and Muller, 1996; Yarden and Schlessinger, 1987a; Yarden and Schlessinger, 1987b). It is now well established that dimerization is not only limited to homodimer formation, but also heterodimerization (Figure 1.3), which is in fact the preferential complex (Brennan et al., 2000; Carraway and Cantley, 1994; Olayioye et al., 2000). The majority of the studies employed cell lines devoid (or expressing very low levels) of endogenous ErbB receptors and were then systematically transfected to express ectopic ErbB receptors either singly or in combination (Alimandi et al., 1997; Cohen et al., 1996; Pinkas-Kramarski et al., 1996b; Riese et al., 1995; Zhang et al., 1996). Heterodimer formation could be detected by co-immunoprecipitation, transphosphorylation, and ligand cross-linking analyses or inferred by the observation of synergistic elevation in Ras-MAPK activity or cellular transformation potential. Similar conclusions regarding receptor dimerization potential were achieved by performing the converse experiments. For example, using intracellular

antibodies that retains ErbB2 in the endoplasmic reticulum thus keeping it from reaching the cell surface, this showed that EGFR and ErbB3 were unable to become phosphorylated following EGF or NRG ligand stimulation, respectively (Beerli et al., 1994; Graus-Porta et al., 1995). More recently, specific inhibition of ErbB3 expression using a designer transcription factor to downregulate *erbB3* expression in ErbB2 overexpressing breast cancer cells dramatically impaired cell proliferation (Holbro et al., 2003). Taken together, the data identified heterodimerization potential leading to varying degrees of signaling co-operativity between ErbB receptors in cell lines.

In addition to the biochemical data identifying receptor combinations, there is also genetic evidence based on gene-targeting studies to indirectly support the heterodimerization model. ErbB2, ErbB4 and the ErbB-ligand, Neuregulin-1 (NRG-1), deficient embryos all die at midgestation due to a ventricular myocardial trabeculation defect (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). These results suggest the formation of ErbB2-ErbB4 heterodimers activated by NRG-1 and are essential for cardiac trabeculation. In this case, the cells expressing the ligand NRG-1 are juxtaposed to the mycocardial cells expressing ErbB2 and ErbB4. In contrast, the ErbB2/ErbB3 heterodimer along with NRG-1 are important for Schwann cell development since *erbB2, erbB3* or *Nrg-1* deficient animals all share the same peripheral nervous system defects (Britsch et al., 1998; Lee et al., 1995; Meyer and Birchmeier, 1995). In the case of the EGFR, genetic studies implicate the formation of both EGFR homodimers and heterodimers. Complete ablation of EGFR in animals result in severe embryonic defects and early death (Sibilia and Wagner, 1995; Threadgill et al., 1995). In

contrast, animals expressing a kinase-defective mutant of EGFR, and unable to form active EGFR homodimers, exhibit only a mild phenotype similar to that of mice deficient for the TGF-α ligand (Fowler et al., 1995; Luetteke et al., 1994; Mann et al., 1993). What this suggests is that EGFR homodimers play a small but important role whereas EGFR heterodimerization with other ErbB receptors either play a major role or can compensate for the defective kinase activity in the EGFR mutant, thus rationalizing the subtle defect.

Although in theory all ErbB receptor dimer combinations are possible, heterodimers are generally more potent than homodimers and there exists a hierarchy for receptor partnerships (Tzahar et al., 1996). It is important to note that under normal conditions, ErbB2 homodimers are less likely to be formed since it is an orphan receptor without a known ligand. This also applies to ErbB3 homodimers since these receptors are kinase defective and its pairing with each other would remain catalytically inert. Moreover, within this network of receptors ErbB2 appears to act as the primary coordinator since it is preferentially recruited by the other ErbB receptors (Graus-Porta et al., 1997). The reason for this preference likely lies in the property of ErbB2 and its potency in effecting its heterodimer partner and potentiating the signaling intensity by decreasing ligand dissociation rates, slowing the rate of downregulation from the surface, and prolonging MAPK signaling (Ben-Levy et al., 1994; Graus-Porta et al., 1995; Karunagaran et al., 1996).

1.5 Structural Aspects of Receptor Activation

1.5.1 The EGFR Extracellular Domain

Recent successes in solving the crystal structures of the extracellular domain of the EGFR (Ferguson et al., 2003; Garrett et al., 2002; Ogiso et al., 2002), ErbB2 (Cho et al., 2003; Garrett et al., 2003), and ErbB3 (Cho and Leahy, 2002) has provided great insight into understanding the structural mechanisms of activation of these receptors. Previous theories speculated that dimerization of the receptors was mediated by the bivalency of EGF (Gullick, 1994; Lemmon et al., 1997; Tzahar et al., 1997) or Heregulin (Tzahar et al., 1997), the so-called "ligand-mediated" dimerization mechanism where the ligand cross-links the receptor pair into a dimer formation. Contrary, the crystal structures showing EGF (Ogiso et al., 2002) or TGFα (Garrett et al., 2002) complexed with the EGFR lead to the same compelling conclusions arguing against the ligand-mediated mechanism. They both rule out the bivalency theory because it is not physically possible for these ligands to span two receptors. Instead, the structural data supports the "receptor-mediated" mechanism for dimerization (Lemmon et al., 1997), which requires two monomeric EGF molecules to bind to two EGFR units in a 2:2 EGF:EGFR complex. The low affinity binding of EGF to EGFR induces a conformational change that releases an intramolecular autoinhibitory interaction and simultaneously exposes a dimerization loop that can mediate its interaction with a neighboring receptor (Ferguson et al., 2003; Garrett et al., 2002; Ogiso et al., 2002). Thus, dimerization is coupled to ligand binding and likewise, ligand binding affinity is influenced by the relative positions of the dimerization loop. For example, truncation of

part of the unit forming the dimerization loop results in at least 10-fold higher affinity for EGF or TGFα than the full length extracellular domain (Garrett et al., 2002). This dimerization loop is highly conserved among all ErbB receptors and may provide the structural paradigm for both homodimerization and heterodimerization (Fleishman et al., 2002). Strikingly, the crystal structure of the extracellular domain of ErbB3 is very similar to that of EGFR (Cho and Leahy, 2002) and is likely to exhibit the same conformational changes upon binding to its ligand heregulin (Figure 1.2). The structural details for the ErbB4 receptor have yet to be resolved.

1.5.2 The ErbB2 Ectodomain

Interestingly, the native structural configuration of the extracellular domain of ErbB2 however is unique. Aside from the unconventional intramembrane ligand, Muc4 (Carraway et al., 1999), there is no defined activating ligand for ErbB2, yet it is the major heterodimerization signaling partner for the other ErbB receptors (Graus-Porta et al., 1997). Indeed, the crystal structure of the ErbB2 ectodomain explains why it cannot bind to any known ligands and reveals that the receptor is already poised in an activated conformation similar to a ligand-bound EGFR (Figure 1.2). The ligand binding domains of ErbB2 are oriented in such a way that it mimics the bridging of the two ligand binding domains of EGFR by an EGF molecule, thus, it spatially occludes any potential ligand recruitment to the receptor (Cho et al., 2003; Garrett et al., 2003). Furthermore, several important residues that are critical for EGFR binding to a ligand are not conserved in the

homologous region of ErbB2 (Garrett et al., 2003). The auto-activation configuration of ErbB2 suggests that the dimerization loop is maintained constitutively in the open conformation and is poised to interact with another receptor, thus by-passing the requirement for a ligand. This does not necessarily imply that ErbB2 receptors readily homodimerize with each other and are obviously not constitutively kinase activated in its wild type form. Overexpression of ErbB2 alone does not lead to oncogenesis. ErbB2 homodimers may be weak and would require a means (such as extreme overexpression or the transmembrane V664E mutation) to bypass the predicted electrostatic repulsion between two ErbB2 receptors (Garrett et al., 2003). Thus, on a structural basis, ErbB2 heterodimerization with other ligand-bound receptors is preferred. To this end, it is of particular clinical interest to determine how an alternate spliced form of ErbB2 identified in primary human breast tumor samples affects the structural configuration of the receptor. The alternate splicing deletes a stretch of residues in the extracellular domain juxtapose the transmembrane domain and results in constitutive catalytic activity.

1.5.3 The Kinase Domain

For ErbB receptors and most other RTKs, the kinase domain remains catalytically inactive until the receptor is ligand bound, thus inducing dimerization and causing the receptor to become autophosphorylated within the activation loop of the kinase region (Hubbard and Till, 2000). This phosphorylation is critical for its enzymatic activity. Prior to ligand binding, the unphosphorylated activation loop in the kinase region forms a conformation that inhibits ATP and substrate from entering the binding pocket and holds

the critical residues in a spatial orientation that is non-functional (Huse and Kuriyan, 2002). This conformation changes dramatically upon phosphorylation of the activation loop. The kinase domain reconfigures itself such that the autoinhibitory interaction is removed and the catalytic region becomes compatible for enhanced phosphotransfer activity. If the specific tyrosine residue in the ErbB2 activation loop is mutated to phenylalanine, this drastically reduces the receptor's ability to phosphorylate a given substrate (Zhang et al., 1998). Other receptors such as the insulin growth factor (IGF) or the fibroblast growth factor (FGF) receptors harbor specific tyrosine residues within the activation loop that are also required to be phosphorylated to achieve complete receptor enzymatic activity (Ellis et al., 1986; Mohammadi et al., 1996). However, the importance of this tyrosine in modulating receptor activity appears to differ. It is not clear if any cytoplasmic proteins participate in potentiating the activation of the receptor, perhaps by acting on the receptor and phosphorylating the activation loop. In contrast to ErbB2, a similar mutation in the EGFR does not alter its kinase activity (Gotoh et al., 1992; Hubbard et al., 1998). Interestingly, the tyrosine kinase region of the EGFR is unique among ErbB receptors because it does not require phosphorylation of its activation loop to promote catalytic activity (Gotoh et al., 1992). Based on the crystal structure, it suggests that the unphosphorylated kinase domain of the EGFR adopts a 'phosphorylated' configuration constitutively without having been phosphorylated (Stamos et al., 2002).

1.6 Receptor Tyrosine Kinase Signaling Specificity

1.6.1 ErbB2 Signaling From the Tyrosine Autophosphorylation Sites

Following activation the receptor undergoes autophosphorylation at several tyrosine residues, which can then act as high affinity docking sites for a number of SH2- or PTB-domain containing proteins. For the ErbB2 receptor, there are five major distinct tyrosine residues within the carboxy-terminal region that have the highest potential to become phosphorylated upon receptor activation. Through a systematic mutagenesis strategy, the ability of individual tyrosine residues to interact with their substrates was assessed. This was achieved by either single mutation of the individual tyrosine residues to the structurally similar phenylalanine amino acid or they were presented in isolation (as an 'add-back' mutant). The significance of each of these sites (Y1028, Y1144, Y1201, Y1226/7, Y1253) in the context of a constitutively activated ErbB2 (rat NeuNT oncogene) was then addressed with respect to cellular transformation and for anchorage independent growth in soft agar assays. Importantly, it was concluded that four of the five autophosphorylation sites appeared to be functionally redundant with respect to cellular transformation. Moreover, these redundant signals are mediated by diverse molecules whose signaling pathways converge at the level of Ras/MAPK activation. Tyrosine Y1144 associates with the Grb2 adaptor protein and Y1226/7 interacts with the Shc signaling protein, both of which can lead to the Ras pathway. The one other autophosphorylation site, Y1028, correlated inversely with ErbB2-mediated transformation and appears to play a negative role in ErbB2 signaling (Dankort et al., 1997; this thesis – Chapter 4).

1.6.2 SH2 Domains

Following ligand-mediated stimulation, receptor oligomerization and kinase activation, tyrosine residues in the cytoplasmic region of the ErbB receptors become phosphorylated. These phosphorylated tyrosine residues then become high affinity binding sites for a number of cytoplasmic or plasma membrane associated proteins involved in transducing the signal to the nucleus. Signaling proteins typically contain modular domains that allow them to directly interact with the receptor according to specificities dictated by the phosphotyrosine residue on the receptor and its surrounding sequences (van der Geer and Pawson, 1995). They have been found to act autonomously, meaning that the same protein interaction domain may be found within different proteins without having any functional similarities. Src homology 2 (SH2) domains are approximately 100 amino acids in length and recognize the canonical phosphotyrosine and the residues that lie 3-5 amino acids carboxy-terminal to it (Eck et al., 1993; Pascal et al., 1994; Waksman et al., 1993). It is these specific C-terminal sequences that provide the discrimination and preferential binding of specific SH2 domains to the phosphotyrosine motif (Songyang et al., 1993; Songyang et al., 1994). Even single amino acid substitutions can alter binding specificity, providing flexibility for the potential roles of the SH2 domains. As an example, the Src SH2 domain may simply be converted to a Grb2-like SH2 domain by substituting a threonine residue to tryptophan (Marengere et al., 1994).

There exists a large family of SH2 domain containing proteins that can be divided into two classes: those with catalytic activity and those without catalytic activity that

function as adapter or docking proteins. SH2 domain-containing proteins possessing intrinsic enzymatic activities include the protein tyrosine kinases (PTK; e.g. Src kinases), SH2-domain phosphatases (SHP1 and SHP2), phospholipase-C (PLCγ) or Ras-GAP. The second group of proteins lack any catalytic activity and only possess distinct modules (SH2, SH3) allowing intermolecular protein interactions. These 'docking' proteins include Grb2, Nck, Crk, and Shc among many others that function to link different proteins involved in signal transduction. One of the best and well known examples of this is exemplified by the role of Grb2 in linking RTKs to the Ras/MAPK signaling cascade. Activated RTKs recruit the Grb2-SH2 domain to a phosphotyrosine site. Grb2 then uses its two SH3 domains to bind to SOS, a Ras guanine-nucleotide exchange factor, which facilitates the GDP-GTP exchange on Ras to produce the active GTP-bound Ras and the subsequent stimulation of the MAPK signaling pathway (Egan et al., 1993; Gale et al., 1993; Li et al., 1993; Lowenstein et al., 1992; Rozakis-Adcock et al., 1993).

1.6.3 PTB Domains

An additional signaling module serving a similar function is the more recently identified phosphotyrosine binding (PTB) domain – also called phosphotyrosine-interacting domains (PID), which consists of approximately 160 residues (Blaikie et al., 1994; Kavanaugh and Williams, 1994; van der Geer and Pawson, 1995). In sharp contrast to SH2 domains, it is the amino acids that are amino-terminal to the

phosphotyrosine residue that confer specificity for PTB domain recognition and binding (Zhou et al., 1995). Moreover, they favour binding to a core NPXpY motif (where N is asparagine; P is proline; X represents any amino acid; pY is the phosphorylated tyrosine), and further preference is determined by hydrophobic residues that lie amino-terminal (-8 to -5 positions relative to pY) to this sequence (Songyang et al., 1995; Trub et al., 1995; van der Geer et al., 1996; van der Geer et al., 1995). The Shc adapter protein, for example, binds to RTK's phosphotyrosine sites via its PTB domain, then becomes tyrosine phosphorylated itself, which generates a suitable docking site for the Grb2-SH2 domain (Ravichandran, 2001). Interestingly, subsequent work has shown that a number of proteins containing PTB domains can bind NPXY motifs in the absence of a phosphorylated tyrosine residue (Borg et al., 1996; Ong et al., 2000). For example, the PTB domain of FRS2 on the one hand recognizes NPXpY on the TrkA receptor, whereas on the other hand it recognizes NPXY on the FGF receptor (Dhalluin et al., 2000). By no means are modular protein binding domains rigid and there likely remains to be discovered variations and other domains or motifs that contribute to the complexity and versatility of protein recognition and binding.

1.6.4 Other Protein-Protein Interactions

SH2 and PTP domains are only two of a growing family of modular protein domains found within intracellular proteins and mediate important protein-protein interactions. For example, two Src homology 3 (SH3) domains flank the SH2 domain in the Grb2 protein and are required to recognize the proline-rich motif in the context of P-

X-X-P sequence (Mayer, 2001) of the Son-Of-Sevenless (SOS) protein. EVH1 and WW domains also recognize proline rich motifs (Fedorov et al., 1999; Macias et al., 1996; Yu et al., 1994). PDZ domains bind to a short amino acid sequence usually located at the far carboxyl end of a protein (Morais Cabral et al., 1996) and EH domains (de Beer et al., 1998) recognize the Asn-Pro-Phe triplet that is usually associated with protein trafficking molecules. Lipid moieties derived from phosphoinositides (PI) are recognized by FYVE and PH domains (Hurley and Meyer, 2001) and are involved in localizing PI kinases to the cell membrane, which is essential for their biological functions. While these domains are obviously of important biological relevance, further discussion about them is beyond the scope of this thesis, however they are reviewed by Pawson and Nash and references within (Pawson and Nash, 2000). Taken together, protein-protein interaction units provide great diversity yet also dictate a high level of specificity to provide unique receptor tyrosine kinase mediated signaling.

1.7 ErbB2 Receptor Substrates

Ligand binding drives RTK dimerization and activation of the cytoplasmic kinase domain followed by autophosphorylation of specific carboxy-terminus tyrosine residues, which become potential docking sites for proteins containing either SH2 or PTB domains. Each ErbB receptor binds to both common and distinct groups of proteins owing to a different number of autophosphorylation sites amongst the receptors, differences in the context of the sequences surrounding the tyrosine residues, and the overall variations in the sequences within the carboxy-terminal regulatory regions

(Olayioye et al., 2000). For example, both the EGFR and ErbB3 have binding sites for the Shc protein, but the EGFR contains three sites for Grb2 binding whereas ErbB3 contains six consensus PI3'K (p85 subunit) binding sites. By using phosphopeptide competition assays, autophosphorylation site mutations, and co-immunoprecipitation assays, this has led to the identification of effector proteins that are responsible for the characteristic biological signals emanating from each receptor. A number of SH2 and PTB proteins that interact with ErbB2 have been documented: PLCγ1 (Fazioli et al., 1991; Peles et al., 1991; Segatto et al., 1992), c-Src (Luttrell et al., 1994; Muthuswamy et al., 1994), Crk (Hempstead et al., 1994), Grb7 (Stein et al., 1994), Chk (Zrihan-Licht et al., 1998), RasGAP (Fazioli et al., 1991), Shc (Dankort et al., 1997; Segatto et al., 1993), Grb2 (Dankort et al., 1997; Janes et al., 1994), Nck (Dougall et al., 1996), DokR (Dankort et al., 2001a), and c-Cbl (Klapper et al., 2000). The precise function and role of some of these proteins in ErbB2 signaling has not been clearly or fully delineated and are currently being pursued.

1.7.1 Grb2 and Shc

Upon further investigation of the specific proteins bound to ErbB2 phosphorylation sites that are involved in mediating the activation of the Ras pathway, it was concluded that the Grb2 adapter protein associates directly with Y1144 while the Shc adapter protein associates directly with Y1227 through its PTB domain (Dankort et al., 1997). Significantly, both the Grb2 and Shc proteins provide a route through which

ErbB2 can stimulate Ras activity (Rozakis-Adcock et al., 1993; Rozakis-Adcock et al., 1992). Grb2 exists in a complex with the guanine nucleotide exchange factor Son of Sevenless (SOS), which can activate Ras by catalyzing the exchange of bound GDP (inactive) for GTP (active) (McCormick, 1994). The recruitment of the Grb2/SOS complex by the activated RTK through binding of the Grb2-SH2 domain to a specific phosphotyrosine site on the receptor carries SOS to the plasma membrane and in close proximity to Ras (Pawson, 1995). Shc proteins are thought to activate Ras indirectly by first recruiting the Grb2-SH2 domain to its phosphotyrosine site, whereas the SH3 domains of Grb2 mediate the Grb2/SOS interaction by recruiting the proline rich region within the carboxy-terminus of SOS (McGlade et al., 1992; Rozakis-Adcock et al., 1992). Thus, this provides the molecular link between ErbB2- Y1144 or Y1227 sites with Ras activation.

1.7.2 c-Src

The c-Src protein tyrosine kinase and its family of related proteins play critical roles in signal transduction (Hunter and Sefton, 1980). Its retroviral oncogenic homolog, v-Src was initially described as a transforming protein derived from avian sarcoma viruses (Varmus et al., 1973) and to date, it is one of the most studied protein tyrosine kinases. Expression of activated c-Src alone is sufficient to induce mammary tumors in mouse models (Webster et al., 1995), where in the absence of c-Src this impairs the ability of mammary tumors to form in animals expressing the Polyomavirus-middleT-antigen (Guy et al., 1994a). Furthermore, elevated expression of c-Src can potentiate the

effects of EGF stimulated responses in cells including DNA synthesis, transformation and turmorigenicity (Luttrell et al., 1988; Maa et al., 1995). However, the role of Src family PTKs in normal ErbB signaling remains unclear. As noted, the c-Src protein kinase (as well as the related c-Yes PTK) can associate directly with ErbB2 (or indirectly with EGFR) leading to the specific activation of c-Src (Luttrell et al., 1994; Muthuswamy and Muller, 1995b; Muthuswamy et al., 1994). Recently, the direct binding site for c-Src in ErbB2 was mapped to a specific tyrosine phosphorylation site within the tyrosine kinase domain of ErbB2. Recapitulation of this binding motif in the homologous region of the EGFR not only led to the direct binding of c-Src with EGFR, but it also resulted in an altered MAPK/Erk activation profile following EGF stimulation as well as causing morphological changes in epithelial cells expressing the c-Src bound EGFR (H. Kim and W.J. Muller, unpublished observations).

1.8 Receptor Downregulation

ErbB receptors may be activated by various ligands and are capable of interacting with distinct complements of intracellular proteins, many of which lead to overlapping signaling pathways. However, the signaling network still maintains the potential to give rise to a broad range of cellular responses. An additional dimension to signal diversification to convey different instructions to the cell depends on the magnitude and duration of a given signal. For example, in PC12 cells a short wave of ERK activation in response to EGF drives cell proliferation whereas NRG drives an extended ERK activation and results in differentiation responses by the cell (Marshall, 1995). This

clearly demonstrates a need to ensure that proper thresholds of signals are reached, maintained and/or attenuated temporally and spatially as necessary. Thus, it is equally important to consider receptor downregulation (Figure 1.4) when discussing the activation of receptor tyrosine kinase signaling.

1.8.1 The c-Cbl E3-Ubiquitin Ligase

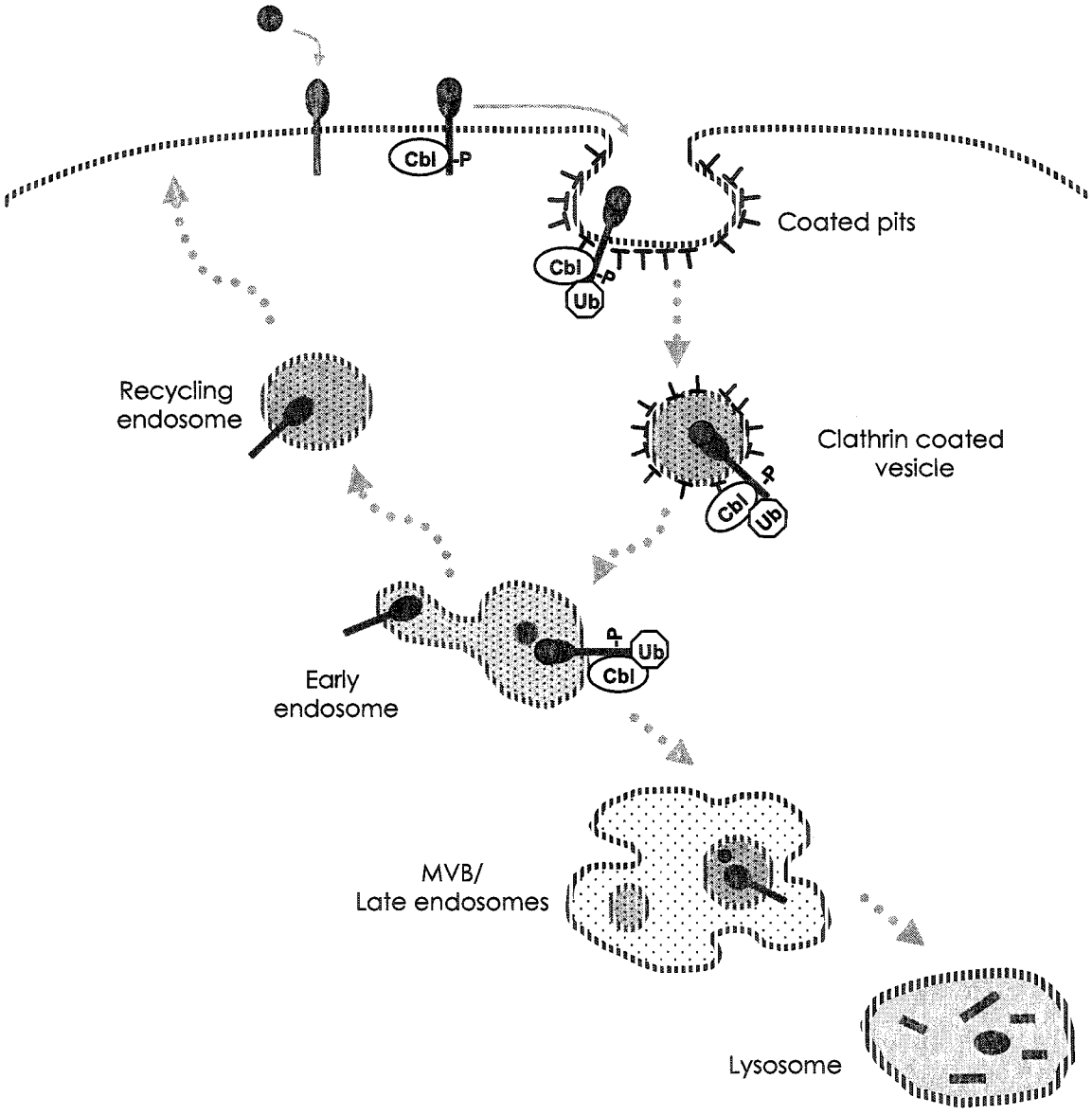
The c-Cbl proto-oncogene was initially identified as the cellular form of v-Cbl, a transforming protein of Cas NS-1 retrovirus that can induce pre- and pro- B-lymphomas and myeloid leukemia (Langdon et al., 1989). It is a relatively large, multivalent protein that is ubiquitously expressed and it primarily lies in the cytoplasm with its highest expression in hematopoietic cells (Thien and Langdon, 2001; Tsygankov et al., 2001). Two additional mammalian members have been identified, Cbl-b and Cbl-3, which make up what is known as the Cbl family, as well as their invertebrate orthologs in *Drosophila melanogater* (D-Cbl) and *Caenorhabditis elegans* (Sli-1) (Hime et al., 1997; Keane et al., 1999; Keane et al., 1995; Meisner et al., 1997; Yoon et al., 1995). All Cbl proteins share a highly conserved amino-terminus referred to as the tyrosine kinase binding (TKB) domain because it can associate with a variety of protein tyrosine kinases. This TKB region includes a four-helix bundle (4H), a calcium-binding EF-hand domain, and most importantly, a variant SH2 domain (Lupher et al., 1996; Meng et al., 1999). Following the TKB region is a short linker region that connects to the RING-finger domain, which acts as an E3-type ubiquitin-protein ligase and mediates the binding of c-Cbl to E2 ubiquitin-conjugating enzymes (Joazeiro et al., 1999; Levkowitz et al., 1999; Yokouchi et

al., 1999). Beyond the RING-finger, the sequences become less conserved amongst the Cbl family. For c-Cbl, the carboxy-terminal half contains a proline-rich region consisting of 15 potential sites of interaction with SH3-domain containing proteins, such as the Grb2 adapter protein (Fukazawa et al., 1995; Meisner et al., 1995; Rivero-Lezcano et al., 1994). In addition to the proline-rich region, there exist several (up to 22) potential tyrosine phosphorylation sites in the carboxy-terminus, which makes c-Cbl a prominent substrate of many PTKs. Tyrosines 700, 731, and 774 appear to be the major phosphorylation sites in c-Cbl that provide docking sites for a variety of SH2-domain containing proteins (Feshchenko et al., 1998).

Evidence that Cbl proteins can regulate tyrosine kinase signaling came from genetic studies in *C.elegans*. Overexpression of Sli-1/Cbl suppressed vulval induction whereas a loss-of-function mutation in Sli-1/Cbl could augment signaling from a weak Let-23/EGFR receptor in vulval development (Yoon et al., 1995). In *Drosophila*, D-Cbl suppressed the development of R7 photoreceptor cells, also a downstream target of EGFR signaling (Meisner et al., 1997). It also has been shown to modulate the dose-sensitive EGFR pathway involved in the dorsal-ventral patterning in oogenesis (Pai et al., 2000). Although it is not a direct link between ErbB receptors and c-Cbl, it is striking to note that c-Cbl deficient mice show increased mammary gland ductal growth with increased density and branching (Murphy et al., 1998). These phenotypes may reflect enhanced ErbB receptor family signaling since they play a prominent role in mammary gland development and their overexpression in mammary glands are associated with hyperplasias and tumor development (Dankort and Muller, 2000).

Figure 1.4 Endocytosis and trafficking of ErbB receptor family

This is a general model (modified from Waterman and Yarden, 2001) primarily based on studies of the EGFR however the behaviour of the other ErbB receptors are similar to varying degrees. The major route of internalization for activated ErbB receptors is through clathrin-coated pits and clathrin-coated vesicles. Uncoating of the clathrin-coated vesicles form the early endosomes, which are then sorted; some recycle back to the cell surface; others are sorted into the inner vesicular structure of the multivesicular bodies (MVB) or late endosomes. These inner vesicles are then delivered to the lysosomal compartment where receptor protein degradation occurs. c-Cbl and receptor ubiquitylation (Ub) are involved in the sorting process by directing the cargo to the lysosomal pathway.


Cbl
P
Coated pits
Cbl
P
Ub
Recycling
endosome
Clathrin coated
vesicle
P
Cbl
Ub
P
Ub
Cbl
Early
endosome
MVB/
Late endosomes
Lysosome

1.8.2 Monoubiquitin

Ubiquitin is a highly conserved 76 amino acid protein that serves as a complex post-translational modification that is conjugated to lysine residues on a number of protein substrates (Weissman, 2001). Ubiquitin itself carries several lysine residues within its sequence that can be self-conjugated to form polyubiquitin chains. In general, protein ubiquitylation has emerged as a versatile regulatory strategy and is an essential process for the degradation of proteins involved in a myriad of processes. The classical view is that proteins that are ubiquitylated with a minimum of four ubiquitin molecules (Chau et al., 1989) to form a ubiquitin chain are targeted for degradation through the ATP-dependent 26S proteosome complex (Deveraux et al., 1994). However, alternative types of ubiquitin modifications are emerging with different consequences for the target protein other than proteosomal degradation. Instead of forming long polyubiquitin chains, some proteins are modified by a single ubiquitin moiety or monoubiquitylated. Monoubiquitin regulates the location and activity of diverse cellular proteins and is involved in three distinct functions: Histone regulation, retroviral budding from the plasma membrane, and endocytosis (Galan and Haguenauer-Tsapis, 1997; Hofmann and Pickart, 1999; Spence et al., 2000; Terrell et al., 1998). It is evident that there is functional diversity with the ubiquitin signal however only its role in endocytosis will be discussed further in detail.

In yeast, the activity of plasma membrane proteins such as the pheromone α-factor receptor (Ste2p) (Hicke et al., 1998) or the G-protein coupled receptor (Terrell et al., 1998) are regulated by ubiquitylation, which targets the protein for downregulation by

internalization. In higher eukaryotes, ubiquitylation-dependent internalization has also been implicated for cell surface proteins including ion channels and signal-transducing receptors (Lee et al., 1999; Levkowitz et al., 1999; Levkowitz et al., 1998; Staub et al., 1997; Strous et al., 1996). The majority of these membrane proteins that are ubiquitylated and endocytosed are subsequently degraded in the lysosomal compartment. One of the better studied examples of this is the Epidermal Growth Factor Receptor. Upon activation of the EGFR by its cognate ligands, the receptor becomes ubiquitylated through the action of the c-Cbl ubiquitin ligase (de Melker et al., 2001; Levkowitz et al., 1998; Longva et al., 2002; Waterman et al., 1999). It was originally thought, based on the smearing pattern observed in ubiquitin immunoblots, that the RTKs were polyubiquitylated, which would mark them for proteosomal degradation. However, the evidence from these studies also suggested that ubiquitylated RTKs were internalized, sorted into late endosomes and multivesicular bodies and finally degraded in the lysosome (Figure 1.4) (Waterman and Yarden, 2001) rather than via the proteosome. This discrepancy was recently reconciled by the use of reagents that could clearly delineate monoubiquitylated versus polyubiquitylated activated RTKs. They concluded that for the EGFR and PDGFR, these receptors are indeed monoubiquitylated at either a single lysine residue or possibly at multiple lysine sites (i.e. multi-ubiquitylated) and that this signal is sufficient for maximal receptor endocytosis and lysosomal degradation (Haglund et al., 2003; Mosesson et al., 2003). They also demonstrate that c-Cbl, although capable of mediating polyubiquitylation of a protein, is limited to the addition of

monomeric ubiquitin to these RTKs (Mosesson et al., 2003). How this process is regulated and prevented from forming polyubiquitin chains is unknown at this time.

Conjugation of a mutant ubiquitin molecule that lacks all of its lysine residues, and therefore cannot be extended into a polyubiquitin chain, can still function normally to promote receptor downregulation. Thus, a single ubiquitin moiety itself carries the internalization signal intrinsically and when appended to an activated receptor, it promotes the internalization of the receptor from the plasma membrane (Nakatsu et al., 2000; Shih et al., 2000). It is also involved in endosomal trafficking to ensure delivery of the receptor from early endosome to late endosome to the lysosomal compartment rather than being recycled to the plasma membrane (Haglund et al., 2003; Katzmann et al., 2001; Raiborg et al., 2002).

1.8.3 Receptor Endocytosis

Cells have different methods to negatively modulate their responses to activated receptor tyrosine kinases in a tightly regulated manner. The link between monoubiquitylated RTKs and the endocytotic route is not very well defined however recent characterization of a family of proteins with an ubiquitin-interacting-motif (UIM) is shedding light on this connection. The current model, although likely over-simplified, suggests that the UIM-containing proteins Eps15, epsin, or Hrs couple monoubiquitylated RTKs to clathrin-coated pits (Raiborg et al., 2002; Shih et al., 2002). Once endocytosed, the early endosomes are delivered to a subset of late endosomes called multivesicular bodies (MVB) because of their multivesicular appearance. Within the MVBs, the

luminal vesicles containing cargo are recognized by the endosomal sorting complex required for transport (ESCRT) (Katzmann et al., 2001). The outer membrane of the MVB then fuses to the lysosomal membrane and subsequently delivers the luminal vesicles to the lysosome where the contents are hydrolytically degraded (Futter et al., 1996). Failure to target the cargo protein to the luminal vesicles leaves the receptor in a predicament where it may be delivered to the lysosome later or likely be recycled back to the plasma membrane.

The EGFR is the prototypical receptor tyrosine kinase, which has been well characterized for the mechanisms to attenuate its activity. For the EGF-EGFR complex, a prominent process to desensitize the receptor is by receptor downregulation, whereby the activated receptors are removed from the cell surface by efficient endocytosis and are shuttled to MVBs (Gorden et al., 1978; Haigler et al., 1979; Miller et al., 1986). From within the MVB, the receptors are sorted to be delivered to the lysosome where they are degraded or recycled back to the cell surface. The significance of this activity is exemplified by expression of an endocytosis impaired EGFR resulting in enhanced mitogenic signaling and transformation (Wells et al., 1990). An inability to downregulate activated receptors can prolong growth factor signaling, which may have deleterious consequences on cellular growth control and development.

The downregulation of the EGFR is facilitated by the addition of ubiquitin molecules to their cytoplasmic domains. Attachment of ubiquitin to the EGFR is coordinated by the ubiquitin ligase activity of c-Cbl and is required for the post-internalization sorting of the EGFR. Overexpression of c-Cbl increases the rate of EGFR

degradation (Thien et al., 2001) whereas the failure to ubiquitylate the EGFR results in the receptors being recycled back to the plasma membrane (Levkowitz et al., 1998). The role of ubiquitin modification appears to be essential for targeting receptors to the multivesicular bodies and subsequently for lysosomal degradation. A kinase deficient EGFR molecule is impaired in its trafficking within the MVB and is not shuttled to the lysosome for degradation (Felder et al., 1990). These results suggest that a kinase activity, and by definition, tyrosine phosphorylation of a target site, are required for sorting the receptor to the MVB pathway. It is possible that an autophosphorylation site in the receptor may be mediating this signal

1.9 Summary of Intent

Tyrosine phosphorylation, which is enzymatically mediated by protein tyrosine kinases, regulates cell proliferation, migration, survival, and differentiation involved in complex biological events. This thesis describes work investigating the function of the tyrosine kinase activity of ErbB2 as well as several of its tyrosine autophosphorylation sites in an in vivo context. In Chapter 3, I establish and validate a targeted *erbB2* cDNA knock-in strategy by demonstrating that expression of a wild type *erbB2* cDNA will rescue the embryonic lethality associated with disruption of the *erbB2* gene. Using the same knock-in strategy, I examined animals expressing a kinase-dead ErbB2 mutant to address the importance of the catalytic activity of ErbB2 in development. Chapter 4 details the characterization of an allelic series of *erbB2* cDNA knock-in animals. I identify a subtle defect in the development of the cutaneous sensory nerves in animals

expressing an ErbB2 receptor decoupled from Shc binding (Y1226/7) but not when the Grb2 binding site (Y1144) was mutated. Additionally, I describe a hypomorphic *erbB2* knock-in allele that was used to establish a minimum threshold level of ErbB2 protein required for normal development. I show that the negative regulatory site, Y1028, plays a role in modulating the level of ErbB2 by promoting the turnover of the receptor. This led to in vitro analyses described in Chapter 5 where I present data identifying the Grb2-mediated recruitment of c-Cbl to ErbB2 but no direct ErbB2/c-Cbl association. Furthermore, ErbB2 appears to be refractory to c-Cbl mediated ubiquitylation. In fact, Y1253 is the critical phosphorylation site required to mediate the monoubiquitylation of ErbB2. Taken together, this thesis describes a number of novel findings that provide important insight into understanding ErbB2 receptor regulation and signaling. The results of my studies may have important implications into the rational design or modification of therapeutic strategies in the treatment of human breast cancer.

Chapter 2

Materials and Methods

Please note that the applicable "Materials and Methods" are described in detail within each chapter.

Table 2.1: List of Methods Used

Chapter 3
Generation of targeting constructs and mutant mice
Ribonuclease Protection Assays
Western blot analyses
Histology
In vitro transcription of Phox2a riboprobes
Chapter 4
Knock-in animals
Immunoblot analysis of embryo lysates
Whole-mount in situ
Immunohistochemistry
Receptor turnover assay
Chapter 5
Plasmids and transfection
Cell culture and cell lysates
Antibodies, immunoprecipitation and immunoblotting

Table 2.2: List of Antibodies Used

Name	Clone	Manufacturer
anti-EGFR	E12020 528	BD Biosciences Santa Cruz
anti-ErbB2	Ab-3 Ab-4	Oncogene Research Products Oncogene Research Products
anti-ErbB3	C-17	Santa Cruz Biotechnology
anti-ErbB4	C-18	Santa Cruz Biotechnology
anti-phosphotyrosine	P11120	BD Biosciences
anti-Grb2	C23	Santa Cruz Biotechnology
anti-c-Cbl	C-15	Santa Cruz Biotechnology
anti-ubiquitin	P4D1 FK1	Santa Cruz Biotechnology Affiniti Research Products
anti-Src	Ab-1	Oncogene Research Products
anti-HA.11	16B12 6908	Babco Sigma
anti-Neurofilament-150	AB1981	Chemicon International
anti-Neurofilament-160	N-5264	Sigma
anti-Bungarotoxin, Alexa 594	B13423	Molecular Probes

Chapter 3

The Catalytic Activity of the ErbB2 Receptor Tyrosine Kinase is Essential for Embryonic Development

Preface

This chapter consists of the article, in the original manuscript format, published previously in *Molecular and Cellular Biology 22: 1073-78 (2002).* The article has been reproduced with permission from the American Society for Microbiology (USA).

To perform structure-function analyses on the ErbB2 receptor tyrosine kinase, in vivo, a targeted knock-in strategy was executed where an *erbB2* cDNA is expressed under the transcriptional control of the endogenous mouse *erbB2* promoter. The validity of this approach is first verified to ensure that the expression of a wild type *erbB2* cDNA can fully rescue the embryonic defects that are known to be associated with genetic disruption of the *erbB2* gene. Next, the effect of expressing a kinase-dead ErbB2 receptor is addressed using the same system. The advantage of this in vivo model is that the behaviour of the mutant receptor is explored in the context of a physiologically significant environment that addresses possible compensatory or overlapping cues and heterodimerization potential with other ErbB receptors. The results clearly demonstrate that the kinase activity of ErbB2 is indispensable and that it is does indeed play a central role within the ErbB receptor family signaling. This sets the precedence for investigating the carboxy-terminal tyrosine autophosphorylation sites as presented in Chapter 4.

The catalytic activity of the ErbB-2 receptor tyrosine kinase is essential for embryonic development.

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Running Title: A kinase-dead ErbB-2 receptor is functionally null.

Keywords: ErbB-2, receptor tyrosine kinase, kinase-dead

***Molecular and Cellular Biology* 22:1073-1078 (2002)**

Abstract

Activation of the epidermal growth factor receptor (EGFR) family is thought to play a critical role in both embryogenesis and oncogenesis. The diverse biological activities of the EGFR family are achieved through various ligand-receptor and receptor-receptor interactions. One receptor that has emerged to play a central role in this signaling network is ErbB-2/Neu and is considered to be the preferred heterodimerization partner for other members of the EGFR family. To assess the importance of the catalytic activity of ErbB-2 in embryonic development, we have generated mice expressing a kinase-dead *erbB-2* cDNA under the transcriptional control of the endogenous promoter. Here, we show that mice homozygous for the kinase-dead *erbB-2* allele are embryonic lethal and display the same spectrum of embryonic defects seen in *erbB-2* knockout mutants. These observations suggest that the catalytic activity of ErbB-2 is essential for normal embryonic development.

Introduction

The EGFR family of growth factor receptor tyrosine kinases (RTK) including ErbB-1/EGFR, ErbB-2/Neu, ErbB-3 and ErbB-4 has been implicated in breast cancer as well as several other human cancers (2). Recently, gene targeting studies have demonstrated specific roles for each of the EGFR family members in normal mammalian development. For example, *erbB-2* (12) and *erbB-4* (6) knockout mice die at midgestation due to deficient cardiac function associated with a lack of myocardial ventricular trabeculation and display abnormal development of the peripheral nervous system. Cardiac rescue of the defects seen in *erbB-2* null mice revealed additional roles for ErbB-2 at the developing neuromuscular junction (13;17). ErbB-3 mutant mice have less severe defects in the heart and consequently are able to survive several days later through embryogenesis. However, sensory and motor neurons in these animals show signs of degeneration due to a lack of proper Schwann cell development (1;22).

Although the structures of the EGFR family receptors have been described in detail, their individual role and contribution to these developmental processes remains to be investigated. To achieve the observed diversity in signaling potential, a coordinate array of ligand-receptor and receptor-receptor interactions is possible. Following activation of the various EGFR family members with one of several EGF family ligands, both homodimeric and/or heterodimeric combinations of receptors are induced and their intrinsic catalytic tyrosine kinase activity is stimulated (30). Upon receptor dimerization, specific tyrosine residues residing in the terminal tail of the receptor dimer become

phosphorylated and serve as important potential binding sites for various intracellular signaling proteins.

Although activated ErbB receptors may partake in any particular combination of homodimerization or heterodimerization complexes, it is important to note that a hierarchical order of preference, stability and signaling potential for each receptor combination is in effect (7). In particular, there is generally a greater preference and likely an advantage for dimerization complexes to include ErbB-2 as the partner because of its potent intrinsic kinase activity. Since no identified ligand is known to bind to and activate ErbB-2 alone, it is considered to be an orphan receptor. Thus, stimulation of ErbB-2 kinase activity may be mediated through normal ligand activation of another ErbB receptor first, which subsequently engages in a specific heterodimer complex (3;18;19). In contrast, ErbB-3 which can bind to and become activated by the ligand neuregulin, is naturally kinase inactive and therefore must depend on a heterodimerization partner for phosphorylation of its tyrosine residues (8;26). Indeed, the ErbB-2:ErbB-3 complex is very stable and transmits a strong mitogenic signal (11). These observations strongly suggest that ErbB-2 plays a central role in the EGFR family signal transduction.

Although genetic ablation studies demonstrate the importance of a receptor to a biological function, it does not address precisely how and which of the individual functional domains of the receptor contributes to the phenotype. It is also unclear within this complex array of receptor dimerization whether the loss of ErbB-2 results in direct or indirect consequences of a lack of the receptor and its interactions with other proteins.

To assess exactly how ErbB-2 may act as the central mediator of the EGFR family of receptors, we investigated whether the kinase activity of ErbB-2 is indeed essential for its complete biological effects. To accomplish this, we generated mice expressing a kinase-dead *erbB-2* cDNA under the transcriptional control of the endogenous *erbB-2* promoter. Mice homozygous for the kinase-dead *erbB-2* mutation died at E10.5 due to a lack of cardiac trabeculation and displayed defects in neural development. These observations argue that the catalytic activity of ErbB-2 is absolutely required for normal embryonic development and cannot be compensated for by other members of the EGFR family.

Materials and Methods

Generation of Targeting Construct and Mutant Mice

Oligonucleotide-directed PCR mutagenesis using the following primers was employed to create the mutant (K757M) kinase-dead ErbB-2 receptor. AB11151: GGA AGT ATA CGA TCG CTA GGC; AB10015: CAC CAT GAT AGC CAC GGG GAT TTT CAC; AB 10014: C GTG GCT ATC ATG GTG TTG AGA GAA AAC; AB 11152: CGA CCT CGG TGT TCT CGG AC. The underlined nucleotides identify the substituted sequence to create the desired mutation. PCR products were subsequently sub-cloned into a wild-type *erbB-2* cDNA and then cloned into the final targeting vector. This plasmid was electroporated into R1 ES cells and G418 or Geneticin (GIBCO-BRL) resistant colonies were picked and subsequently screened by Southern Blot analysis for correctly targeted mutants. Mutant mice were generated from the positive ES cell clones

by method of blastocyst injection into balb/c derived blastocysts (10). Subsequent generations were maintained in an SV129/Balb/c background.

RNase Protection Assays

RNA was extracted and purified from individual embryos by the Guanidine Isothyiocyanate-Cesium Chloride (GiT-CsCl) method as previously described (27). For RNase protection assays, as described previously (24), 30 μg of total RNA was used and hybridized to an anti-sense *erbB-2* riboprobe (23). The protocol was modified by lowering the overnight hybridization to 45°C and only T1 RNase (450 units) was used in the digestion reactions for 20 minutes at 37°C.

Western Blot Analysis

Fresh or flash frozen embryos were lysed in TNE lysis buffer (24). Cleared lysates were electrophoresed through SDS-polyacrylamide gels and the proteins transferred to PVDF membranes (Immobilon-P; Millipore). The membranes were blocked in 10% skim milk/TBS for 1 hour at room temperature and then incubated with the appropriate antibody. For ErbB-2 immunoblots, the membranes were incubated with anti-ErbB-2 antibodies (1:1000; AB-3, Oncogene Science) overnight at 4°C. Immunoblots for Grb2 were performed with rabbit anti-Grb2 polyclonal sera (1:2500; C-23, Santa Cruz). After the primary antibody incubations, membranes were subjected to 4 x 15 minute washes in TBS-1% Tween-20 (Bio-Rad). Subsequently, horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5000; Jackson

Laboratories) were incubated with the membranes for 1 hour at room temperature then washed 2 x 15 minutes in TBS-1% Tween-20 and 2 x 15 minutes in TBS alone. Immunoblots were visualized by enhanced chemiluminescence (Amersham) as specified by the manufacturer.

Histology

Embryos from timed-matings were dissected free from the placenta and cleared of extraembryonic tissues. A small piece of the visceral yolk sac was retained and placed in tail lysis buffer (100 mM Tris-HCl, pH 8.5; 5 mM EDTA; 0.2% SDS; 200 mM NaCl; 100 μg/μl Proteinase K), the DNA isolated and subsequently used for genotyping the embryos. Dissected embryos were quickly rinsed in ice-cold PBS and transferred directly into 4% paraformaldehyde, fixed overnight at 4°C, washed 2x in 70% ethanol and stored at 4°C in 70% ethanol. For standard hematoxylin and eosin (Fisher Scientific) staining, samples were embedded in paraffin and 8μm serial sections were cut and mounted. For whole-mount *in situ* hybridizations, embryos were fixed in 4% paraformaldehyde/0.2% glutaraldehyde (Fisher Scientific), dehydrated through a graded series of methanol/PBT (1X PBS/0.1% Tween-20, Sigma) baths and stored in 100% MeOH at -20°C until needed. Whole-mount *in situ* hybridizations were carried out as previously described (29).

In vitro transcription of Phox2a riboprobes

The Phox2a riboprobe plasmid, pKS903 SSN (25), was digested with SstII and

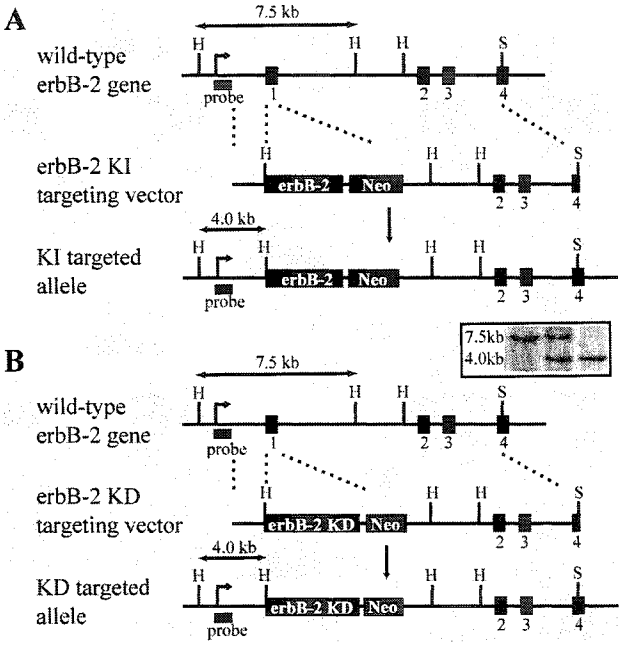
transcribed with the T3 RNA polymerase to generate an antisense riboprobe; the sense riboprobe was generated using the same template, but linearized with HindIII and transcribed with T7 RNA polymerase. The *in vitro* transcription reactions were carried out in 20µl reaction volumes containing 14 µl dH_2O (DEPC), 2 µl 10X transcription buffer (Boehringer Mannheim), 2 µl DIG RNA Labelling Mix (Boehringer Mannheim), 1 µg of template DNA, 30 units RNAGuard (Pharmacia) and 30 units of RNA polymerase (Boehringer Mannheim or Gibco-BRL). The reactions were incubated @ 37°C for 2 hours and stopped with the addition of 20 units of DNaseI (RNAse-Free, Boehringer Mannheim). The riboprobes were precipitated and resuspended in 100 µl of DEPC dH_2O (~100 ng/µl).

Results and Discussion

A kinase-dead (KD) variant of the ErbB-2/Neu receptor was created by oligonucleotide-directed PCR mutagenesis to generate a point mutation affecting lysine residue 757. This K757M alteration ablates the conserved ATP-binding lysine residue in the tyrosine kinase domain resulting in its inability to phosphorylate its substrates (20;21;28). We have confirmed that disruption of this key amino acid results in ablation of ErbB-2 associated kinase activity and also inactivates the potent transforming activity of an oncogenic *erbB-2* mutant (data not shown). To determine the functional importance of the ErbB-2 kinase activity *in vivo*, we generated a targeting vector in which the first coding exon of the endogenous *erbB-2* gene was replaced with either a wild-type *erbB-2* cDNA (*erbB-2* knock-in or KI) (Figure 1A) or a cDNA harboring the K747M mutation

Figure 1. Targeted *erbB-2* cDNA knock-in strategy by homologous recombination

For germline expression, a targeting vector was constructed where exon 1 was replaced by either (**A**) a wild-type *erbB-2* cDNA or (**B**) a cDNA encoding the kinase-dead *erbB-2* mutation, followed by a PGK-neomycin (Neo) cassette and targeted to the endogenous *erbB-2* locus by homologous 5' and 3' flanking arms. Digestion of genomic DNA with HindIII (H) and subsequent Southern Blot analysis (inset) with an external probe, as indicated, resulted in a 7.5 kb band for the endogenous allele whereas the knock-in cDNA alleles introduced a HindIII site and resulted in a 4.0 kb band.


A
7.5 kb
wild-type
erbB-2 gene
probe
erbB-2 KI
targeting vector
erbB-2
Neo
4.0 kb
KI targeted
allele
B
7.5kb
4.0kb
erbB-2 KD
KD targeted
allele
H
S

(*erbB-2* KD) (Figure 1B). To facilitate recovery of targeted recombination events, a PGK-Neo expression cassette was inserted downstream of the inserted cDNA. The constructs were electroporated into R1 ES cells and independent clonal lines were isolated and subjected to Southern blot analyses with an appropriate external probe to identify successful targeting events (Figure 1).

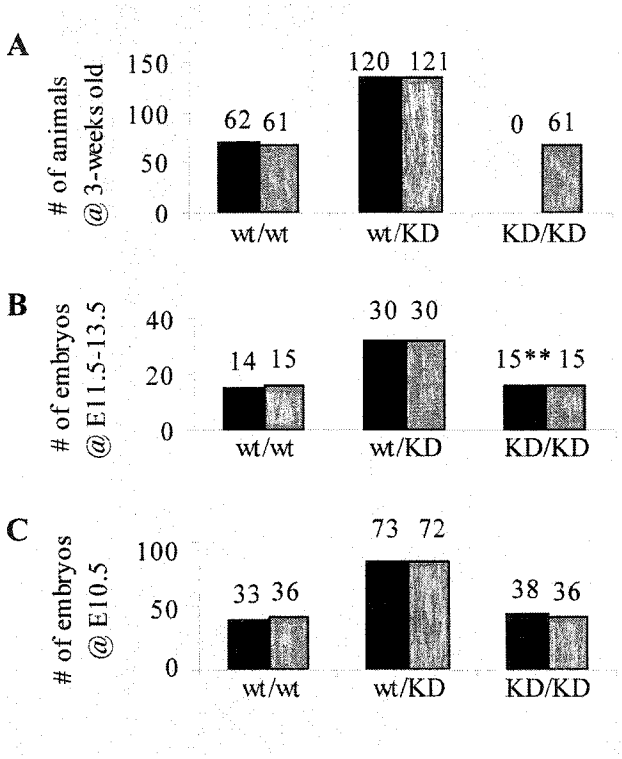
After microinjection of several independently targeted ES cell lines into donor blastocysts, chimeric mice were obtained and bred to identify those that transmitted the mutant alleles through the germline. *ErbB-2*$^{wt/KI}$ and *erbB-2*$^{wt/KD}$ mice appeared normal and were fertile. *ErbB-2*$^{KI/KI}$ mice were also viable and were generated at the expected Mendelian ratios (data not shown). Thus, in contrast to the generation of *erbB-2*$^{-/-}$ mice where exon 1 was replaced by a PGK-neomycin cassette (12), replacement of the first coding exon of *erbB-2* with a wild-type *erbB-2* cDNA rescued the embryonic lethality associated with disruption and inactivation of the *erbB-2* gene. Interestingly, no viable *erbB-2*$^{KD/KD}$ mutant mice were observed in the litters generated from heterozygous matings that were subsequently genotyped at 3-weeks of age (Figure 2A).

Since *erbB-2*$^{-/-}$ embryos died at midgestation due to defects in heart development (12), we assessed whether we could detect viable embryos at E10.5 for our kinase-dead mutants. To accomplish this, timed matings between heterozygous animals were set up and embryos were dissected from the uterus and observed. Embryo genotypes were determined by analysis of DNA isolated from their respective visceral yolk sacs. At E10.5 each possible genotype was present at the expected Mendelian frequencies (Figure 2C) and all embryos were viable, possessed a heartbeat and appeared normal in size.

Figure 2. Embryonic lethality at midgestation in kinase-dead mutants

Mendelian ratios from the progeny of heterozygous matings were determined and compared with the frequency of genotypes observed. (**A**) No homozygous *erbB-2*$^{KD/KD}$ mutant animals were observed at weaning age (3-weeks old). (**B**) Observations at E11.5-E13.5 revealed the expected number of mutant embryos however all *erbB-2*$^{KD/KD}$ (**) embryos were being resorbed and no heartbeat was detected. (**C**) At E10.5, all the embryos appeared healthy and were present in proportion with Mendelian frequencies.

(■ Observed, ▩ Expected)


A
# of animals @ 3-weeks old
150
100
50
0
62 61
120 121
0 61
wt/wt
wt/KD
KD/KD
B
# of embryos @ E11.5-13.5
40
20
0
14 15
30 30
15** 15
wt/wt
wt/KD
KD/KD
C
# of embryos @ E10.5
100
50
0
33 36
73 72
38 36
wt/wt
wt/KD
KD/KD

However, in mutant embryos their hearts were slightly enlarged and had irregular heartbeats. Further observations at E11.5-E13.5 (Figure 2B) revealed that homozygous mutant *erbB-2*$^{KD/KD}$ embryos, although present at the expected frequency, had no heartbeat and showed signs of resorption such as arrested growth, pale color, and soft tissue. Thus, consistent with the embryonic lethality of *erbB-2*$^{-/-}$ mutants, these observations confirmed that mutant embryos expressing the kinase-dead ErbB-2 receptor were dying *in utero* at midgestation between E10.5 and E11.5.

To investigate the cause of embryonic lethality, we performed histological analyses of E10.5 embryos. Both *erbB-2*$^{KI/KI}$ (Figure 3A) and *erbB-2*$^{wt/KD}$ (Figure 3B) knock-in embryos were completely normal in their development of the heart trabeculae. In contrast, *erbB-2*$^{KD/KD}$ mutant embryos clearly lacked development of ventricular trabeculae (Figure 3C) that likely resulted in the observed reduction of embryonic blood flow. These results are strikingly consistent with the defects previously identified in *erbB-2*$^{-/-}$ embryos as well as in *neuregulin*$^{-/-}$ and *erbB-4*$^{-/-}$ embryos (6;12;15). Thus, the cardiac defects seen in the kinase-dead mutants were directly attributable to a loss of ErbB-2's enzymatic tyrosine kinase activity.

We also examined whether the same peripheral nervous system abnormalities seen in *erbB-2*$^{-/-}$ mutants were also similarly affected by expression of the kinase-dead mutation. One important marker for peripheral neural tissues is the Phox-2a transcription factor (16). To explore whether neural development was perturbed in the KD mice, embryos derived from E10.5 and E11.5 were subjected to wholemount *in situ* analyses using an anti-sense Phox2a riboprobe (Figure 4). In both *erbB-2*$^{wt/wt}$ (Figures 4A & B)

Figure 3. Defects in heart development in E10.5 mutant embryos

Parasaggital sections of (**A**) *erbB-2*$^{KI/KI}$, (**B**) *erbB-2*$^{WT/KD}$ and (**C**) *erbB-2*$^{KD/KD}$ embryos at E10.5 were stained with hematoxylin and eosin. Although heartbeats were detected at the time of dissection, histological examinations of the hearts revealed a lack of trabeculae in the ventricles of *erbB-2*$^{KD/KD}$ mutants but were present in heterozygous littermates and in age-matched *erbB-2*$^{KI/KI}$ knock-in embryos.

t, trabeculae; v, ventricle; ec, endocardial cushion; a, atrium.

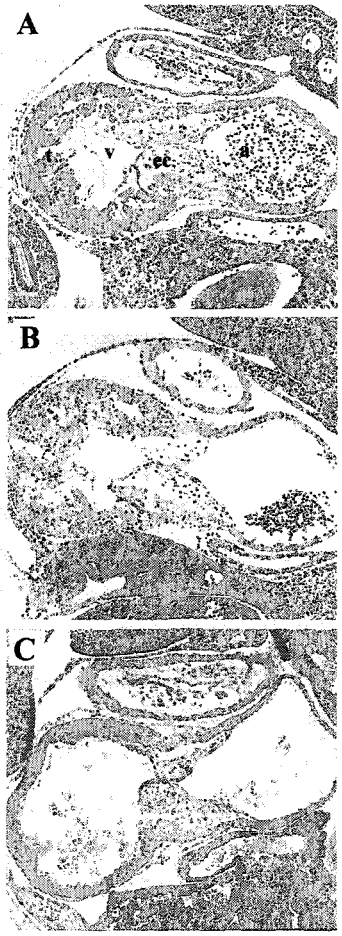
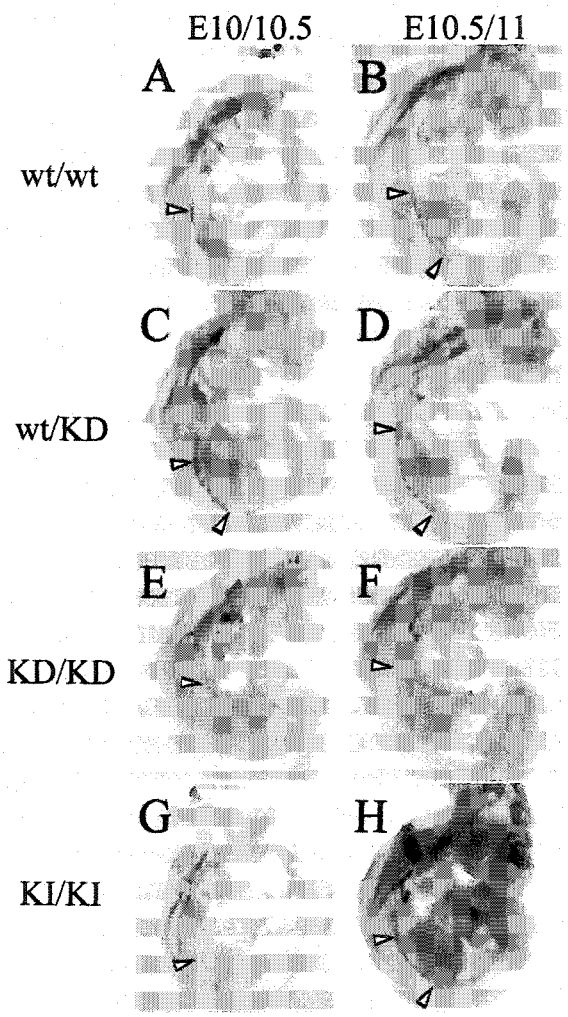

A
v
ec
B
C

Figure 4. Lack of sympathetic chain ganglia in kinase-dead mutants

Day E10/E11 embryos were subjected to wholemount *in situ* hybridization analysis using an anti-sense Phox-2a riboprobe. Normal sympathetic chain ganglia development was present in (**A & B**) *erbB-2*$^{WT/WT}$ embryos and (**C & D**) *erbB-2*$^{WT/KD}$ heterozygous embryos, whereas (**E & F**) *erbB-2*$^{KD/KD}$ homozygous mutants lacked proper development or they were delayed in (**G & H**) *erbB-2*$^{KI/KI}$ embryos. The white arrowheads highlight the developing sympathetic chain ganglia

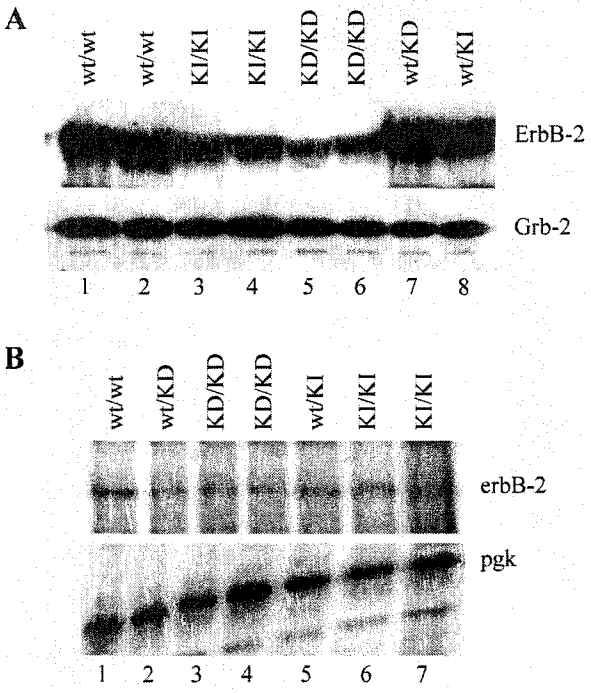

E10/10.5
E10.5/11
A
B
wt/wt
C
D
wt/KD
E
F
KD/KD
G
H
KI/KI

and *erbB-2*$^{wt/KD}$ (Figures 4C & D) embryos, the sympathetic chain developed normally. However, in the E10.5/11 *erbB-2*$^{KD/KD}$ homozygous embryos, only a weak Phox2a signal could be detected in the rostral-most regions of the sympathetic chain, a clear indication that this structure had either failed to initiate development or was incapable of developing in the kinase-dead genetic background (Figures 4E & F). Analyses of Phox2a expression in *erbB-2*$^{KD/KD}$ (Figure 4E) and *erbB-2*$^{KI/KI}$ (Figure 4G) embryos revealed that the sympathetic chain appeared to be absent at E10/10.5 when compared to age-matched wild-type and heterozygous embryos (compare Figure 4G with Figure 4A & C). However, by E10.5/11 a lengthy sympathetic chain had developed in the *erbB-2*$^{KI/KI}$ homozygotes (Figure 4H) but remained completely absent in the *erbB-2*$^{KD/KD}$ embryos (Figure 4F). These observations indicate that the catalytic activity of ErbB-2 is also essential for normal development of the primary sympathetic chain ganglia.

To confirm that these knock-in alleles expressed ErbB-2, we performed Western immunoblot (Figure 5A) and Ribonuclease (RNase) protection (Figure 5B) analyses. As shown in Figure 5B, the wild-type *erbB-2* KI allele and the kinase-dead allele expressed similar levels of *erbB-2* transcripts. Similarly, immunoblot detection for ErbB-2 also revealed comparable protein levels between the two knock-in mutants at E10.5 (Figure 5A). The slightly lower levels of ErbB-2 seen in the *erbB-2*$^{KD/KD}$ embryos relative to *erbB-2*$^{KI/KI}$ embryos is likely due to and consistent with the loss of tissue structures in the kinase-dead mutants (Figures 2 and 3) that would normally express significant levels of ErbB-2. Interestingly, both the *erbB-2*$^{KI/KI}$ and the *erbB-2*$^{KD/KD}$ knock-in embryos expressed only 10-15% of the expected ErbB-2 protein as detected in *erbB-2*$^{wt/wt}$ embryos

Figure 5. Detection of ErbB-2 expression in E10.5 embryos

Expression of ErbB-2 generated from the cDNA insert in *erbB-2*^{KIKI} and *erbB-2*$^{KD/KD}$ embryos was determined. For (**A**) immunoblot analyses, total protein isolated from E10.5 embryos were subjected to SDS-PAGE and incubated with an anti-ErbB-2 antibody. Detection of Grb-2 (lower panel) protein was used to control for equal protein lysate quantification. (**B**) RNase protection assays with an anti-sense *erbB-2* riboprobe were employed to detect *erbB-2* transcripts in total RNA isolated from *erbB-2*$^{wt/wt}$ (lane 1), *erbB-2*$^{KD/KD}$ (lanes 3 & 4), and *erbB-2*$^{KI/KI}$ (lanes 6 & 7) embryos. A mouse phosphoglycerate kinase (PGK) riboprobe (lower panel) was used as an internal control for equal sample loading in each lane.


A
wt/wt
wt/wt
KI/KI
KI/KI
KD/KD
KD/KD
wt/KD
wt/KI
ErbB-2
Grb-2
1 2 3 4 5 6 7 8
B
wt/wt
wt/KD
KD/KD
KD/KD
wt/KI
KI/KI
KI/KI
erbB-2
pgk
1 2 3 4 5 6 7

(Figure 5A, compare lanes 1, 2 to 3-6) despite expressing comparable levels of *erbB-2* transcripts (Figure 5B) which may reflect a requirement for splicing events for efficient transport and translation of the mRNA. In this regard it should be noted that embryos expressing both copies of the wild-type knock-in *erbB-2* allele display a 24-hour delay in development of the sympathetic chain (Figure 4) suggesting that reduced ErbB-2 levels may have a minor phenotypic consequence.

Our observations have important implications in understanding the role of *erbB-2* in promoting both normal cardiac and neural development. Previous studies have demonstrated that the integrity of ErbB-2, ErbB-4 or Neuregulin is essential for the development of the cardiac trabecular extensions. Despite the presence of a functional ErbB-4 protein that could potentially transphosphorylate ErbB-2, our results further suggest that the catalytic activity of ErbB-2 alone is required to recapitulate the necessary signal transduction pathways leading to proper trabeculation. In contrast to myocardial trabeculae formation, ErbB-2 and ErbB-3 are thought to mediate the survival of neural crest cells contributing to the development of the peripheral nervous system since similar cranial nerve phenotypes were seen in mutant ErbB-2 and mutant ErbB-3 mice as well as in Neuregulin mutants (12;15;22). Given that ErbB-3 is kinase defective and is completely dependent on its heterodimerization partners for its activation (8), inactivation of ErbB-2 catalytic activity would be expected to have profound effects on neural development. Taken together, we can conclude that ErbB signaling is dependent on the kinase activity of ErbB-2 and that a kinase-dead ErbB-2 mutant acts essentially as a functionally null receptor.

In contrast to the embryonic lethality caused by inactivation of the catalytic activity of ErbB-2, a naturally occurring germline mutation in the kinase domain of EGFR known as Waved-2 are completely viable and only display epithelial defects such as a wavy hair phenotype (5;14). Thus, unlike ErbB-2, other EGFR family members can presumably compensate for the severe impairment in EGFR catalytic activity. The difference between these phenotypes may reflect the hierarchical importance of ErbB-2 within the EGFR family signaling network. Alternatively, the difference in the phenotype in these strains may reflect the fact that the Waved-2 mutation has not completely ablated the catalytic activity of EGFR (14) and thus retains a higher degree of biological function.

Given the dominant-negative action of the kinase-dead ErbB-2 receptor expressed *in vitro* (Qian et al., 1994a) it is surprising that mice heterozygous for the *erbB-2* KD allele failed to exhibit any obvious phenotype that might be expected of a trans-dominant inhibition of the remaining wild-type allele. One potential explanation for this observation is that the level of kinase-dead ErbB-2 is insufficient to interfere with the remaining endogenous wild-type ErbB-2 receptor. Indeed, the *erbB-2* KD allele produced only 10% of the expected ErbB-2 protein (Figure 5). To exclude this possibility, we have crossed *erbB-2* KI mice with *erbB-2* KD mice since both these knock-in alleles expressed similar levels of ErbB-2. Phenotypic analyses of these crosses demonstrated that the *erbB-2* KD allele failed to exhibit a dominant-negative effect on the remaining *erbB-2* KI allele (data not shown). Thus, like the Waved-2 EGFR kinase mutation the *erbB-2* KD

allele fails to exhibit any discernable dominant-negative effect on the remaining intact ErbB-2 receptor.

Studies with other receptor tyrosine kinase (RTK) have concluded that the catalytic activity of RTK is dispensable for normal physiological function of the receptor. For example, the Flt-1 (VEGF receptor) null mutation resulted in early embryonic lethality at E8.5 with disorganized blood vessels (4). However, mice expressing a kinase-deficient Flt-1 survived and showed normal angiogenesis (9), suggesting that other components of the receptor are more important to its functional role. In contrast to these observations, we have found that the catalytic activity of ErbB-2 is essential for embryonic development. The embryonic lethality associated with the expression of the kinase-dead ErbB-2 is not likely due to inappropriate localization of the receptor since it has previously been demonstrated that this mutant receptor is efficiently expressed on the cell surface (20). Given that ErbB-2 is the preferred heterodimerization partner for the other EGFR family members (7), the requirement for ErbB-2 catalytic function *in vivo* suggest that its catalytic activity is critical for EGFR family signaling. Future studies with these strains should allow for identification of downstream targets of ErbB-2.

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References

1. **Britsch, S., L. Li, S. Kirchhoff, F. Theuring, V. Brinkmann, C. Birchmeier, and D. Riethmacher**. 1998. The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. Genes Dev **12**:1825-1836.

2. **Burden, S. and Y. Yarden**. 1997. Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. Neuron **18**:847-855.

3. **Carraway, K.L. and L.C. Cantley**. 1994. A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. Cell **78**:5-8.

4. **Fong, G.H., J. Rossant, M. Gertsenstein, and M.L. Breitman**. 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature **376**:66-70.

5. **Fowler, K.J., F. Walker, W. Alexander, M.L. Hibbs, E.C. Nice, R.M. Bohmer, G.B. Mann, C. Thumwood, R. Maglitto, and J.A. Danks**. 1995. A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. Proc Natl Acad Sci U S A **92**:1465-1469.

6. **Gassmann, M., F. Casagranda, D. Orioli, H. Simon, C. Lai, R. Klein, and G. Lemke**. 1995. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature **378**:390-394.

7. **Graus-Porta, D., R.R. Beerli, J.M. Daly, and N.E. Hynes**. 1997. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J **16**:1647-1655.

8. **Guy, P.M., J.V. Platko, L.C. Cantley, R.A. Cerione, and K.L. Carraway**. 1994. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc Natl Acad Sci U S A **91**:8132-8136.

9. **Hiratsuka, S., O. Minowa, J. Kuno, T. Noda, and M. Shibuya**. 1998. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc Natl Acad Sci U S A **95**:9349-9354.

10. **Joyner, A.L.** 1993. Gene Targeting - A Practical Approach. Oxford University Press,

11. **Karunagaran, D., E. Tzahar, R.R. Beerli, X. Chen, D. Graus-Porta, B.J. Ratzkin, R. Seger, N.E. Hynes, and Y. Yarden**. 1996. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. EMBO J **15**:254-264.

12. **Lee, K.F., H. Simon, H. Chen, B. Bates, M.C. Hung, and C. Hauser**. 1995. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature **378**:394-398.

13. **Lin, W., H.B. Sanchez, T. Deerinck, J.K. Morris, M. Ellisman, and K.F. Lee**. 2000. Aberrant development of motor axons and neuromuscular synapses in erbB2-deficient mice. Proc Natl Acad Sci U S A **97**:1299-1304.

14. **Luetteke, N.C., H.K. Phillips, T.H. Qiu, N.G. Copeland, H.S. Earp, N.A. Jenkins, and D.C. Lee**. 1994. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. Genes Dev **8**:399-413.

15. **Meyer, D. and C. Birchmeier**. 1995. Multiple essential functions of neuregulin in development. Nature **378**:386-390.

16. **Morin, X., H. Cremer, M.R. Hirsch, R.P. Kapur, C. Goridis, and J.F. Brunet**. 1997. Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene Phox2a. Neuron **18**:411-423.

17. **Morris, J.K., W. Lin, C. Hauser, Y. Marchuk, D. Getman, and K.F. Lee**. 1999. Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. Neuron **23**:273-283.

18. **Pinkas-Kramarski, R., M. Shelly, B.C. Guarino, L.M. Wang, L. Lyass, I. Alroy, M. Alimandi, A. Kuo, J.D. Moyer, S. Lavi, M. Eisenstein, B.J. Ratzkin, R. Seger, S.S. Bacus, J.H. Pierce, G.C. Andrews, Y. Yarden, and M. Alamandi**. 1998. ErbB tyrosine kinases and the two neuregulin families constitute a ligand-receptor network. Mol Cell Biol **18**:6090-6101.

19. **Pinkas-Kramarski, R., L. Soussan, H. Waterman, G. Levkowitz, I. Alroy, L. Klapper, S. Lavi, R. Seger, B.J. Ratzkin, M. Sela, and Y. Yarden**. 1996. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. EMBO J **15**:2452-2467.

20. **Qian, X., W.C. Dougall, M.E. Hellman, and M.I. Greene**. 1994. Kinase-deficient neu proteins suppress epidermal growth factor receptor function and abolish cell transformation. Oncogene **9**:1507-1514.

21. **Qian, X., C.M. LeVea, J.K. Freeman, W.C. Dougall, and M.I. Greene**. 1994. Heterodimerization of epidermal growth factor receptor and wild-type or kinase-deficient Neu: a mechanism of interreceptor kinase activation and transphosphorylation. Proc Natl Acad Sci U S A **91**:1500-1504.

22. **Riethmacher, D., E. Sonnenberg-Riethmacher, V. Brinkmann, T. Yamaai, G.R. Lewin, and C. Birchmeier**. 1997. Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature **389**:725-730.

23. **Siegel, P.M., D.L. Dankort, W.R. Hardy, and W.J. Muller**. 1994. Novel activating mutations in the neu proto-oncogene involved in induction of mammary tumors. Mol Cell Biol **14**:7068-7077.

24. **Siegel, P.M., E.D. Ryan, R.D. Cardiff, and W.J. Muller**. 1999. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. EMBO J **18**:2149-2164.

25. **Valarche, I., J.P., M.R. Hirsch, S. Martinez, C. Goridis, and J.F. Brunet**. 1993. The mouse homeodomain protein Phox2 regulates Ncam promoter activity in concert with Cux/CDP and is a putative determinant of neurotransmitter phenotype. Development **119**:881-896.

26. **Wallasch, C., F.U. Weiss, G. Niederfellner, B. Jallal, W. Issing, and A. Ullrich**. 1995. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. EMBO J **14**:4267-4275.

27. **Webster, M.A., J.N. Hutchinson, M.J. Rauh, S.K. Muthuswamy, M. Anton, C.G. Tortorice, R.D. Cardiff, F.L. Graham, J.A. Hassell, and W.J. Muller**. 1998. Requirement for both Shc and phosphatidylinositol 3' kinase signaling pathways in polyomavirus middle T-mediated mammary tumorigenesis. Mol Cell Biol **18**:2344-2359.

28. **Weiner, D.B., Y. Kokai, T. Wada, J.A. Cohen, W.V. Williams, and M.I. Greene**. 1989. Linkage of tyrosine kinase activity with transforming ability of the p185neu oncoprotein. Oncogene **4**:1175-1183.

29. **Wilkinson, D.G. and M.A. Nieto**. 1993. Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. Methods Enzymol. **225**:361-373.

30. **Yarden, Y. and A. Ullrich**. 1988. Growth factor receptor tyrosine kinases. Annu Rev Biochem **57**:443-478.

Chapter 4

Modulation of ErbB2 Signaling During Development: A Threshold Level of ErbB2 Signaling is Required for Development

Preface

The manuscript presented in Chapter 4 was submitted to the journal *Development* on March 10th, 2004 and we are currently waiting for their reviews and its status for publication.

Upon receptor activation, the catalytic activity of the tyrosine kinase domain is stimulated and acts upon tyrosine residues in the carboxy-terminus of the receptor. Phosphorylation of these tyrosine sites creates potential binding sites for interaction with downstream effector molecules. Thus, it is logical to follow the study of the ErbB2 catalytic domain described in Chapter 3 with an investigation into the roles of some of the tyrosine autophosphorylation sites. Based on the information provided by the previously published in vitro studies on ErbB2-Y1028, Y1144, and Y1226/7, I describe in this chapter the characterization of knock-in mice generated using the exact same strategy used in Chapter 3, to express the ErbB2-Y1028F, Y1144F, or Y1226/7F mutant receptors. The results presented provide novel insight into several facets of ErbB2 signaling and function in development. The study also led to the realization of a minimum threshold level of ErbB2 that is required for development and raises interest into the regulatory mechanisms upon ErbB2.

Modulation of ErbB2 signaling during development: A threshold level of ErbB2 signaling is required for development

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Running Title: **Threshold level of ErbB2 in Development**

Summary

We have generated a series of *erbB2* cDNA knock-in animals to explore the role of signaling pathways coupled to ErbB2 during development. Although this knock-in allele was hypomorphic expressing 10-fold less ErbB2 protein than wild type, the knock-in animals were healthy. However, a further 2-fold reduction in ErbB2 levels in hemizygous knock-in animals resulted in perinatal lethality with defects in the innervation of the diaphragm. Genetic rescue of this hypomorph was accomplished by expression of the ErbB2-Y1028F mutant in a comparable knock-in allele. Interestingly, hemizygous Y1028F animals were viable with normal innervation of the diaphragm. Molecular analyses revealed that the Y1028F allele expressed higher levels of ErbB2 and that Y1028 promoted the turnover of the receptor. In addition, ablation of the Shc binding site in ErbB2 (Y1227) resulted in subtle defects in the sensory nerves. Thus, we have established how ErbB2 levels may be modulated through development and that a minimum threshold level of ErbB2 is required.

Introduction

The ErbB2 receptor tyrosine kinase is a member of the Epidermal Growth Factor Receptor (EGFR) family, which also includes the EGFR/ErbB1, ErbB3, and ErbB4. Although there is considerable homology amongst the EGFR family members, each EGFR family member can interact with a distinct subset of ligands (Olayioye et al., 2000; Pinkas-Kramarski et al., 1996a; Yarden and Sliwkowski, 2001). For example, EGFR ligands such as EGF or TGFβ bind directly to the EGFR whereas the heregulins are specific ligands for ErbB3 or ErbB4 (Carraway et al., 1994; Peles et al., 1993). In contrast to these EGFR family members, a direct soluble ligand for ErbB2 has yet to be described. However, there have been several reports suggesting that a member of the Mucin family known as Muc-4 may act as a membrane bound ligand for ErbB2 (Carraway et al., 1999).

Despite the lack of a direct soluble ligand for ErbB2, the tyrosine kinase activity of ErbB2 can be stimulated by other EGFR ligands through the formation of specific heterodimers with other members of the EGFR family (Goldman et al., 1990; Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996a; Pinkas-Kramarski et al., 1996b; Tzahar et al., 1996). For example, stimulation of cells with EGF can result in transphosphorylation of ErbB2 through the formation of EGFR-ErbB2 heterodimers (King et al., 1988; Stern and Kamps, 1988). Similarly, stimulation of cells with heregulin can result in the transphosphorylation of ErbB2 through the formation of specific heterodimers of ErbB2 and ErbB4 or heterodimers of ErbB2 and ErbB3 (Goldman et al., 1990; Karunagaran et al., 1996; Pinkas-Kramarski et al., 1998a; Pinkas-Kramarski et al., 1996a; Pinkas-Kramarski et al., 1998b; Pinkas-Kramarski et al., 1996b; Sliwkowski et al., 1994; Stern and Kamps,

1988; Tzahar et al., 1997; Tzahar et al., 1996). Transphosphorylation of ErbB3 is absolutely dependent on its capacity to form specific heterodimers with other members of the EGFR family since it lacks intrinsic tyrosine kinase activity (Guy et al., 1994b). Taken together, these observations argue that the formation of specific heterodimers play an important biological role in the function of the EGFR family.

The EGFR family share structural similarities including an extracellular ligand binding domain, a single pass transmembrane domain, a highly conserved tyrosine kinase domain, and a regulatory carboxyl-terminal tail containing several tyrosine autophosphorylation sites (Hynes and Stern, 1994). Ligand mediated activation results in strong mitogenic signals from the receptors leading to cellular growth and differentiation. Not surprisingly, members of the EGFR family are collectively involved in both development and in disease. Whereas amplification and aberrant overexpression of ErbB2 has been implicated in various cancers, most notably in breast cancer (Simpson et al., 1995; Slamon et al., 1987; Slamon et al., 1989), the loss of *erbB2, erbB3 or erbB4* expression in knock-out mice has deleterious effects on the developing embryo (Britsch et al., 1998; Gassmann et al., 1995; Lee et al., 1995; Riethmacher et al., 1997). For example, *erbB2* and *erbB3* deficient animals share similar hypoplastic development of the sympathetic nervous system (Britsch et al., 1998) and *erbB2* and *erbB4* deficient animals exhibit defective formation of cardiac ventricular trabecules (Gassmann et al., 1995; Lee et al., 1995).

Upon receptor activation, specific tyrosine residues in the terminal tail of the receptor are autophosphorylated (Akiyama et al., 1991; Hazan et al., 1990), which then

serve as potential binding sites for intracellular signaling proteins harboring phosphotyrosine binding (PTB) (Kavanaugh et al., 1995) or Src homology 2 (SH2) (Pawson, 1995) domains. In a series of studies by Dankort et al. (1997), the five major tyrosine autophosphorylation sites (Y1028, Y1144, Y1201, Y1227, Y1253) were evaluated systematically for their roles in constitutively activated ErbB2-mediated transformation of fibroblast cells. Although simultaneous ablation of all five sites in the tyrosine phosphorylation deficient (NYPD) mutant drastically impaired transformation, independent tyrosine-to-phenylalanine mutations at four of five sites only modestly reduced the transforming ability. Substitution at the one remaining site (Y1028) resulted in a consistent increase in transformation. Conversely, restoration of individual tyrosine residues to the NYPD mutant at one of four sites ("add-back" mutants) was not only able to fully restore the transforming ability, but it was also able to transform with a modest increase over the fully functional receptor. The Y1028 add-back mutant had the opposite effect and completely ablated the residual transforming abilities of the NYPD mutant. These observations suggested that while four of the five tyrosine sites positively mediate ErbB2 signaling, Y1028 negatively modulates ErbB2 activity.

We describe here the introduction of wild type and phosphotyrosine mutant *erbB2* cDNAs into the endogenous mouse *erbB2* locus to examine their physiological roles, *in vivo*. Although mice derived from the different knock-in alleles were viable, examination of the levels of ErbB2 expression revealed that the knock-in strains expressed only 10% of the expected ErbB2 protein. A further reduction of ErbB2 protein, achieved by intercrossing the *erbB2* knock-in mutants with *erbB2* null mice, resulted in perinatal

lethality. Thus, we established a minimum threshold level of ErbB2 required for viability. In contrast, expression of the Y1028F mutation in knock-in animals genetically rescued this perinatal lethality. Biochemical analyses revealed that these animals expressed higher levels of ErbB2 above the threshold. We further identify that Y1028 mediates the negative regulation of ErbB2 signaling by influencing the turnover rate of the receptor.

Results

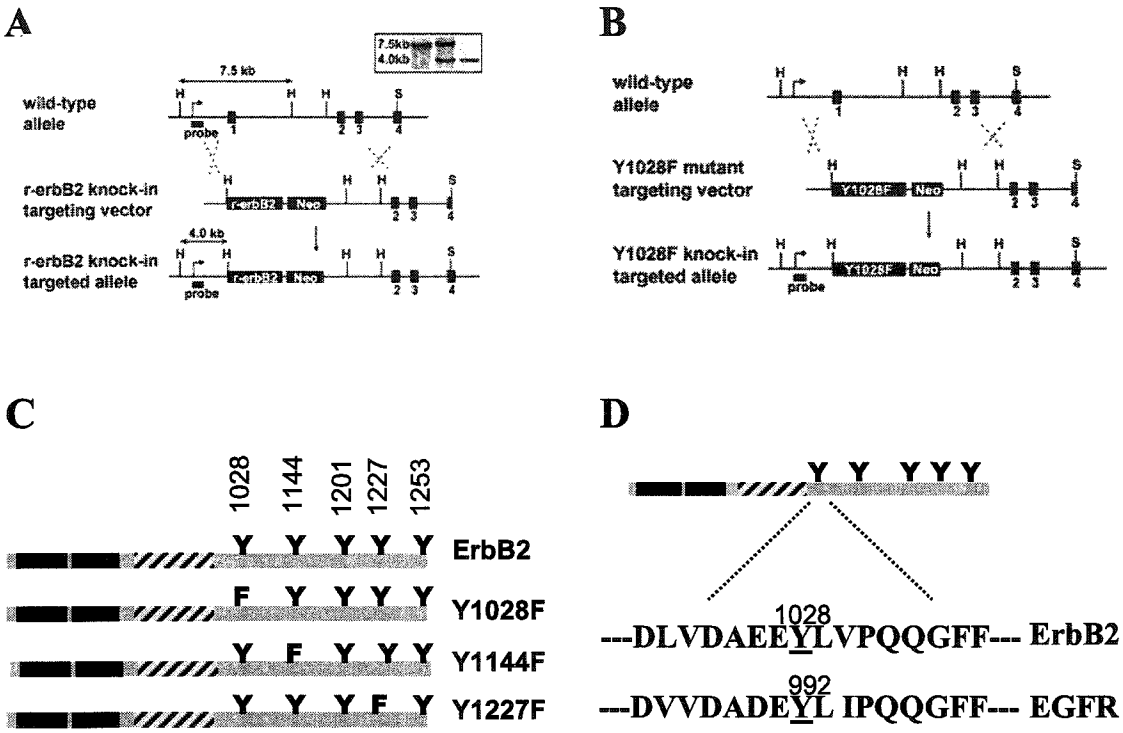
Generation of erbB2 cDNA knock-in animals

To assess the relative contribution of the different ErbB2 tyrosine autophosphorylation sites, we employed a targeted knock-in strategy involving replacement of the first coding exon of the mouse *erbB2* gene (*m-erbB2*) with the rat *erbB2* cDNA (*r-erbB2*) (Figure 1A). Thus, expression of *m-erbB2* is disrupted and replaced by expression of the *r-erbB2* cDNA under the control of the endogenous promoter. For genotyping purposes, note that the '*wt*' superscript (e.g. $erbB2^{wt}$) will be used herein to designate the endogenous mouse *erbB2* genotype whereas the '*erbB2*' superscript (e.g. $erbB2^{erbB2}$) will be used to designate the knock-in *r-erbB2* cDNA allele. Following germline transmission of the targeted knock-in allele, transgenic animals homozygous for the *erbB2* knock-in cDNA ($erbB2^{erbB2/erbB2}$) were viable, appeared healthy, and reproduced at the expected Mendelian frequencies (Table 1).

To examine the functional roles of the tyrosine autophosphorylation sites *in vivo*, mutant *r-erbB2* cDNA harboring single tyrosine-to-phenylalanine substitutions (Figure

Figure 1. Generation of knock-in animals expressing ErbB2 tyrosine phosphorylation mutants.

The knock-in targeting vector was constructed such that exon 1 of the mouse *erbB2* gene was replaced with either (**A**) a rat *r-erbB2* cDNA or (**B**) a cDNA encoding a mutant ErbB2 receptor harboring the Y1028F mutation. The cDNA is followed by a PGK-Neomycin/SV40-polyA expression cassette and is targeted to the endogenous *erbB2* locus by homologous 5' and 3' flanking arms, placing it directly under the transcriptional control of the endogenous *erbB2* promoter. The targeted allele introduces an additional HindIII site that was used to distinguish between the endogenous wild type allele (7.5kb fragment) and the targeted allele (4.0kb fragment) in Southern Blot analyses (**A, inset).** (**C**) Schematic representation of the ErbB2 receptor depicted with the five tyrosine autophosphorylation sites in the C-terminal tail and with the corresponding amino acid number. Note that the numbering of the amino acids is based on the rat ErbB2 sequence and will be referred to herein by these designates. Also shown are the three individual tyrosine-to-phenylalanine mutations described in this report: Y1028F, Y1144F and Y1227F. (**D**) Alignment of the amino acid sequences surrounding tyrosine 1028 in ErbB2 and tyrosine 992 in EGFR. (**E**) Expression analysis of the knock-in allele. Total protein lysates prepared from E12.5 wild type and *erbB2* cDNA knock-in embryos were used to detect ErbB2 levels. Detection of Grb2 protein (lower panel) served as an internal loading control.


A
wild-type allele
r-erbB2 knock-in targeting vector
r-erbB2 knock-in targeted allele
7.5 kb
4.0 kb
probe
Neo
B
wild-type allele
Y1028F mutant targeting vector
Y1028F knock-in targeted allele
Y1028F
Neo
probe
C
1028
1144
1201
1227
1253
ErbB2
Y1028F
Y1144F
Y1227F
D
1028
---DLVDAEEYLVPQQGFF--- ErbB2
992
---DVVDADEYL IPQQGFF--- EGFR
E
wt/wt
wt/erbB2
wt/erbB2
erbB2/erbB2
erbB2/erbB2
IB: ErbB2
IB: Grb2
1 2 3 4 5

1C) were also expressed in mice using an identical targeting strategy as described above but substituting the cDNA cassette (Figure 1B). Specifically, we generated knock-in mice expressing the ErbB2-Y1028F mutant, as well as the ErbB2-Y1144F mutant (loss of the direct binding site for Grb2) and the ErbB2-Y1227F mutant (loss of the direct binding site for Shc). Homozygous *erbB2*$^{Y1028F/Y1028F}$ knock-in animals expressing the Y1028F mutation were generated from heterozygous matings at the expected Mendelian frequencies (Table 1) and appeared normal and healthy. Interestingly, homozygous *erbB2*$^{Y1144F/Y1144F}$ and *erbB2*$^{Y1227F/Y1227F}$ knock-in mice were also normal, healthy and fertile (Table 1).

The use of a rat *erbB2* cDNA instead of a mouse cDNA was necessary to distinguish expression of the knock-in allele from the endogenous gene and allow for the validation of the knock-in strategy. The mouse and rat *erbB2* share a greater than 93% sequence similarity at both the nucleic acid and amino acid levels. Importantly, the tyrosine autophosphorylation sites and the sequences surrounding these sites in the carboxyl-terminus are conserved. To confirm that rescue of the embryonic lethality associated with disruption and inactivation of the *m-erbB2* gene (Morris et al., 1999) was attributed solely to expression of the knock-in *r-erbB2* cDNA, RT-PCR was performed on total RNA isolated from wild type and knock-in animals. Analysis of the RT-PCR product suggested that homozygous animals indeed only expressed the *r-erbB2* cDNA (data not shown).

Hypomorphic expression of ErbB2 protein resulted in neonatal lethality.

Since specific expression of the knock-in allele was validated, we next performed immunoblot analyses on protein lysates prepared from E12.5 wild type, heterozygous, and homozygous mutant embryos (Figure 1E) to determine whether the knock-in *erbB2* allele expressed wild type levels of ErbB2 protein. Surprisingly, the levels of ErbB2 expressed in the $erbB2^{erbB2/erbB2}$ embryos (Figure 1E, lanes 4-5) were dramatically reduced relative to the expression of endogenous ErbB2 in wild type littermates (Figure 1E, lanes 1-2). However, in spite of the considerably reduced levels of ErbB2 protein, homozygous $erbB2^{erbB2/erbB2}$ knock-in animals did not display any obvious phenotype and appeared generally healthy.

Although mice bearing the knock-in *r-erbB2* cDNA appeared phenotypically normal, we have previously demonstrated that they indeed exhibited subtle defects. In particular, our lab showed that *r-erbB2* cDNA knock-in animals possessed only 10% the number of muscle spindles compared to wild type animals (Andrechek et al., 2002). Consequently, we investigated the effects of further reducing the expression of ErbB2 by interbreeding heterozygous knock-in ($erbB2^{wt/erbB2}$) animals with heterozygous *erbB2* knock-out ($erbB2^{wt/ko}$) animals. This strategy allowed us to express a single *erbB2* knock-in allele in an *erbB2* deficient background to generate hemizygous $erbB2^{erbB2/ko}$ animals. Significantly, no $erbB2^{erbB2/ko}$ animals were found at weaning age (3-weeks old) when the animals were genotyped (Table 1).

To determine the precise reason that the $erbB2^{erbB2/ko}$ animals were dying, we interbred homozygous $erbB2^{erbB2/erbB2}$ knock-in animals with heterozygous $erbB2^{wt/ko}$

Table 1: Enumeration of the different genotypes at 3-weeks and at birth

Parental Genotype	# of animals at 3-weeks of age		
	Wild type	**Heterozygous**	**Mutant**
$erbB2^{wt/erbB2}$ x $erbB2^{wt/erbB2}$	35	74	31 *(erbB2/erbB2)*
$erbB2^{wt/Y1028F}$ x $erbB2^{wt/Y1028F}$	47	103	49 *(Y1028F/Y1028F)*
$erbB2^{wt/Y1144F}$ x $erbB2^{wt/Y1144F}$	40	79	36 *(Y1144F/Y1144F)*
$erbB2^{wt/Y1227F}$ x $erbB2^{wt/Y1227F}$	36	70	33 *(Y1227F/Y1227F)*
$erbB2^{wt/erbB2}$ x $erbB2^{wt/ko}$	29	60	0 *(erbB2/ko)*
$erbB2^{wt/Y1028F}$ x $erbB2^{wt/ko}$	31	61	26 *(Y1028F/ko)*

Parental Genotype	# of animals at birth / # of survivors	
	Heterozygous	**Mutant**
$erbB2^{erbB2/erbB2}$ x $erbB2^{wt/ko}$	20/20	18/0 *(erbB2/ko)*
$erbB2^{Y1144F/Y1144F}$ x $erbB2^{wt/ko}$	13/13	13/0 *(Y1144F/ko)*
$erbB2^{Y1227F/Y1227F}$ x $erbB2^{wt/ko}$	12/12	11/0 *(Y1227F/ko)*

animals and assessed whether we could detect viable hemizygous *erbB2*$^{erbB2/ko}$ animals at birth. Although the expected number of animals were present at birth, 18 of the 38 newborn pups were either still born or started dying immediately after birth (Table 1). They could not breathe independently despite the ability to open their mouths and they became cyanotic and died within a few minutes. Indeed, all of the dead pups were genotyped to be *erbB2*$^{erbB2/ko}$ animals. Subsequent postmortem histological analyses of the lungs confirmed that the hemizygous *erbB2*$^{erbB2/ko}$ pups were unable to inflate and expand their lungs, despite being vascularized and structurally intact (data not shown). These observations suggest that a critical minimal threshold level of ErbB2 protein is required to maintain viability.

Expression of the Y1028F mutation rescues the hypomorph

Previous *in vitro* analyses of the tyrosine phosphorylation mutants identified tyrosine 1028 as a negative regulator of ErbB2-induced transformation via an undetermined mechanism. Accordingly, we next asked whether we could genetically rescue the perinatal lethality observed with the hemizygous *erbB2*$^{erbB2/ko}$ animals by removing the putative negative regulatory tyrosine residue in ErbB2. This was accomplished by similarly crossing the heterozygous *erbB2*$^{wt/Y1028F}$ knock-in animals with *erbB2*$^{wt/ko}$ animals to generate mice expressing a single *Y1028F* cDNA knock-in allele in an *erbB2*-deficient background. Interestingly, in contrast to the hemizygous *erbB2*$^{erbB2/ko}$ pups, no perinatal lethality was observed with the hemizygous *erbB2*$^{Y1028F/ko}$ animals (Table 1). In fact, all of the progeny survived and the adult *erbB2*$^{Y1028F/ko}$

animals appeared normal, healthy and were fertile. To preclude any possibility that the phenotypic differences observed in the ErbB2-Y1028F knock-in animals versus the control *erbB2* knock-in animals were not by chance and that it is specifically due to this particular point mutation, mice expressing either the Y1144F or the Y1227F phosphotyrosine mutant ErbB2 receptor in an *erbB2*-deficient background were also generated. As indicated in Table 1, none of the hemizygous $erbB2^{Y1144F/ko}$ or $erbB2^{Y1227F/ko}$ animals survived and similar to the $erbB2^{wt/ko}$ animals, they died shortly after birth struggling unsuccessfully to breathe.

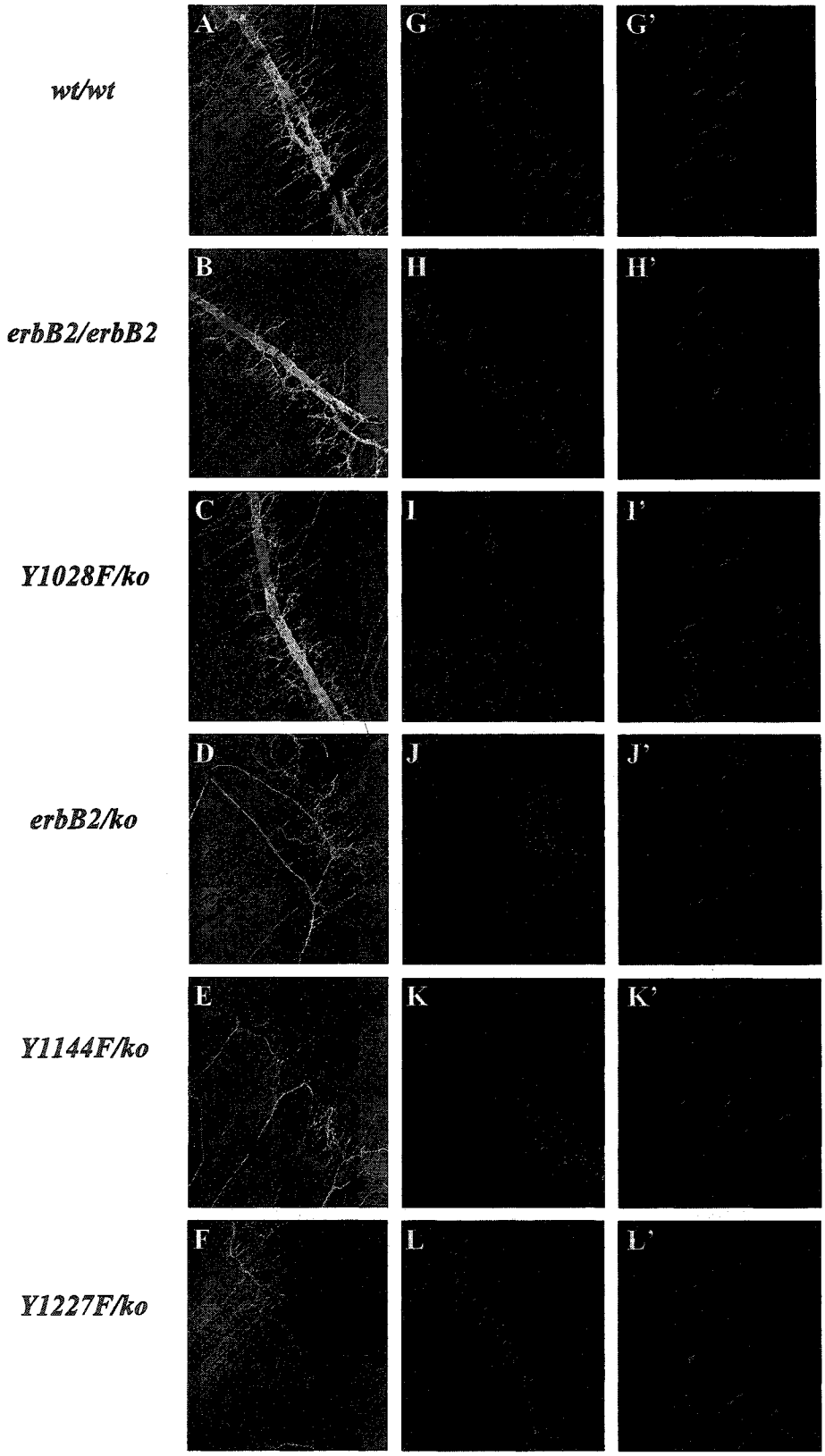
To further elucidate the nature of the defect resulting in the inability of the animals to inflate their lungs, the neuromuscular junctions in the diaphragm muscles of E18.5 embryos were examined by whole-mount immunostaining with a neurofilament-150 antibody and with α-bungarotoxin. In the control animals, $erbB2^{wt/wt}$ and $erbB2^{erbB2/erbB2}$, the central region of the diaphragm muscles were innervated by the relatively large main phrenic nerve with smaller intramuscular branches (Figures 2A & B). Accordingly, innervation of the diaphragm from the genetically rescued embryos, $erbB2^{Y1028F/ko}$, were similar to the control animals (Figure 2C). In contrast, mutant diaphragms from $erbB2^{erbB2/ko}$, $erbB2^{Y1144F/ko}$, $erbB2^{Y1227F/ko}$ embryos were very poorly innervated, which likely resulted in the perinatal respiratory failure. As shown in Figures 2D, E, and F, the innervations were thin, disorganized, and discontinuous or incomplete and they lacked an apparent main nerve trunk from which smaller presynaptic branches originate. However, labeling of postsynaptic acetylcholine receptor (AChR) clusters with

Figure 2. Expression of the Y1028F mutant genetically rescues the hypomorphic knock-in allele.

Whole-mount diaphragm muscle from E18.5 embryos were stained with neurofilament antibodies to label presynaptic axons and with α-bungarotoxin-Alexa594 to label AChRs. (**A-C**) The main central trunk of the phrenic nerve in diaphragm muscle isolated from (**A**) $erbB2^{wt/wt}$ (**B**) $erbB2^{erbB2/erbB2}$ and (**C**) $erbB2^{Y1028F7/ko}$ are shown. The clusters of AChR correspond to the path of the presynaptic axon shown at low power magnification and at higher magnification (**G'-I'**). These particular genotypes are healthy animals and do not exhibit the acute respiratory distress at birth.

(**D-E**) In contrast, the animals that were unable to inflate their lungs (**D**) $erbB2^{erbB2/ko}$ (**E**) $erbB2^{Y1144F/ko}$ and (**F**) $erbB2^{Y1227F/ko}$ had poorly innervated diaphragms where the phrenic nerves were thinned, defasciculated and fragmented. However, the density and shape of the AChR clusters appeared to be unaffected in these animals (**J-L** and **J'-L'**).

(Original magnification: 100X for A-L and 400x for G'-L')


wt/wt
erbB2/erbB2
Y1028F/ko
erbB2/ko
Y1144F/ko
Y1227F/ko
A
B
C
D
E
F
G
H
I
J
K
L
G'
H'
I'
J'
K'
L'

α-bungarotoxin did not reveal any significant differences in the number, density or shape of AChR clusters (Figures 2G-L, G'-L').

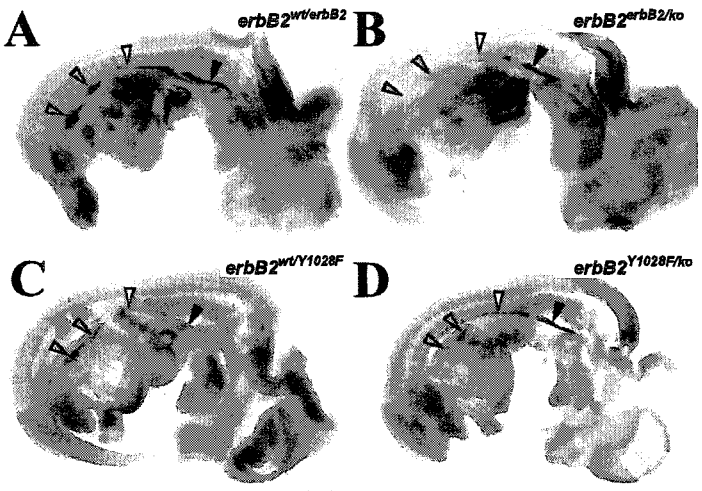
Additional evidence for the genetic rescue of the hypomorphic *erbB2* knock-in allele by expression of the Y1028F mutation was observed in the developing sympathetic nervous system, where ErbB2 plays a critical role. For comparison, Figures 3A and 3C show the normal development of the primary sympathetic chain ganglia in heterozygous embryos at E12.5, using Phox2a as a marker. In $erbB2^{erbB2/ko}$ embryos, there was only partial development of the thoracic sympathetic ganglia and the defects became more severe in a rostral-caudal gradient (Figure 3B). In comparison, the sympathetic ganglia of $erbB2^{Y1028F/ko}$ embryos were similar in size and length as its heterozygous littermates (Figure 3D). Taken together, these results strongly suggest that ErbB2-Y1028 specifically mediates a negative regulatory effect on the receptor and this plays an important function *in vivo* to modulate the activity of the receptor throughout development.

The Y1028F mutation maintains higher ErbB2 protein levels

Since we hypothesized earlier that a minimal threshold level of ErbB2 is required for normal development, we explored whether this Y1028F mutation resulted in increased levels of ErbB2 such that the hemizygous animals survived. To determine the molecular basis for the hypermorphic $erbB2^{Y1028F}$ allele, immunoblot analyses were performed on protein lysates derived from E12.5 embryos. Expression of the Y1028F receptor mutant in the $erbB2^{Y1028F}$ knock-in animals consistently resulted in significantly

Figure 3. Development of the primary sympathetic ganglion chain.

Whole-mount in situ hybridization staining of the sympathetic nervous system using a Phox2a antisense riboprobe on E12.5 embryos. Mid-saggital views of (**A**) $erbB2^{wt/erbB2}$, (**B**) $erbB2^{erbB2/ko}$, (**C**) $erbB2^{wt/Y1028F}$, (**D**) $erbB2^{Y1028F/ko}$. Black arrowheads point to the superior cervical ganglia; White arrowheads highlight the thoracic sympathetic chain ganglia; Green and Blue arrowheads indicate the cells that migrate from the caudal portion of the primary sympathetic chain to the mesentery or the anlage of the adrenal gland. Original magnification of A through F is 16X.

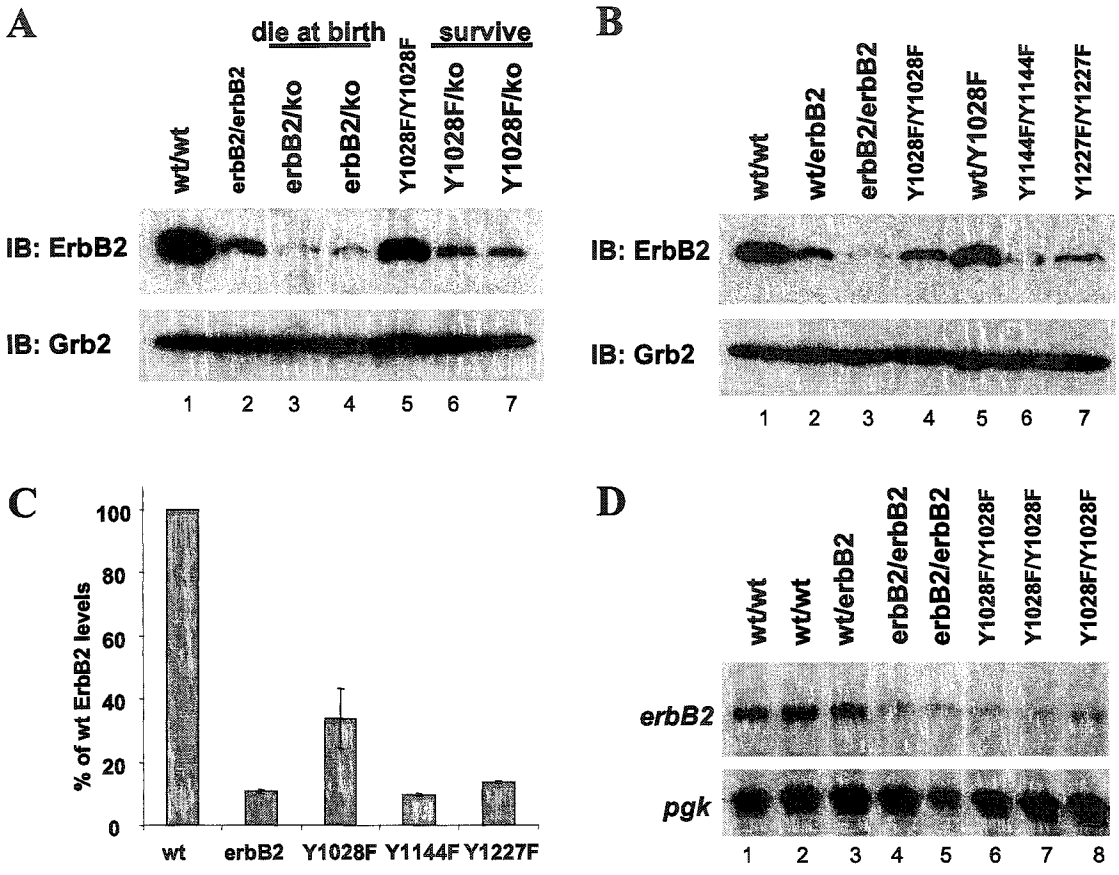

A
erbB2wt/erbB2
B
erbB2erbB2/ko
C
erbB2wt/Y1028F
D
erbB2Y1028F/ko

higher levels of ErbB2 (Figure 4A, lane 2 vs. 5, lanes 3-4 vs 6-7; Figure 5B, lane 3 vs. 4). In the hemizygous embryos, there is a critical difference in ErbB2 levels in the *erbB2*$^{Y1028F/ko}$ embryos (Figure 4A, lanes 6-7) versus the *erbB2*$^{erbB2/ko}$ embryos (Figure 4A, lanes 3-4), which likely determined the difference whether the animals survived or died shortly after birth. Note that the ErbB2 levels in *erbB2*$^{Y1028F/ko}$ embryos (Figure 4A, lanes 6-7) are comparable to the levels in *erbB2*$^{erbB2/erbB2}$ embryos (Figure 4A, lane 2), which are essentially healthy animals.

To confirm that the observed increase in ErbB2 protein is specific to expression of the *Y1028F* allele, we also examined the levels of ErbB2 in homozygous E12.5 embryos expressing either the ErbB2-*Y1144F* mutation or the ErbB2-*Y1227F* mutation. The results revealed that only embryos with the *Y1028F* allele expressed elevated levels of ErbB2 when compared to the levels observed in knock-in embryos expressing the *erbB2* cDNA, the *Y1144F* cDNA, or the *Y1227F* cDNA alleles (Figure 4B, compare lane 4 vs. 3, 6, 7). In fact, quantitative immunoblotting using I^{125}-conjugated secondary antibodies and subsequent ImageQuant (Molecular Dynamics) analyses revealed that the levels of ErbB2 in *erbB2*$^{Y1028F2/Y1028F}$ embryos were at least 3-8-fold higher than in *erbB2*$^{erbB2/erbB2}$, *erbB2*$^{Y1144F/Y1144F}$ and *erbB2*$^{Y1227F/Y1227F}$ embryos (Figure 4C). Thus, the quantitative difference in ErbB2 levels alone likely determined the difference between animals surviving or dying at birth.

In order to assess whether the variation in ErbB2 protein levels observed in the *erbB2* or *Y1028F* knock-in alleles were occurring at a transcriptional or post-transcriptional level, *erbB2* transcripts in E12.5 embryos were detected using an RNase

Figure 4. Comparison of ErbB2 protein and transcript levels in knock-in embryos. For immunoblot analyses, total protein was isolated from the different E12.5 embryos and subjected to SDS-PAGE. Where possible, control littermates were used for comparison analyses. Membranes were subsequently incubated with an anti-ErbB2 antibody (**A & B,** upper panel). Detection of Grb2 protein (**A & B,** lower panel) was used as a sample loading control. (**C**) ErbB2 protein levels were quantified by using ^{125}I-conjugated secondary antibodies and analyzed using PhosphoImager and ImageQuant software. The absolute levels of ErbB2 detected were normalized to Grb2 levels. The graph depicts relative levels of ErbB2 expressed as a percentage of ErbB2 levels in wild type embryos. (**D**) *erbB2* transcript levels (upper panel) were detected by RNase protection assays on total RNA isolated from E12.5 embryos. The mouse phosphoglycerate kinase (*pgk*) riboprobe (lower panel) was used as an internal control for equal sample loading.


A
die at birth
survive
wt/wt
erbB2/erbB2
erbB2/ko
erbB2/ko
Y1028F/Y1028F
Y1028F/ko
Y1028F/ko
IB: ErbB2
IB: Grb2
1 2 3 4 5 6 7
B
wt/wt
wt/erbB2
erbB2/erbB2
Y1028F/Y1028F
wt/Y1028F
Y1144F/Y1144F
Y1227F/Y1227F
IB: ErbB2
IB: Grb2
1 2 3 4 5 6 7
C
% of wt ErbB2 levels
100
80
60
40
20
0
wt
erbB2
Y1028F
Y1144F
Y1227F
D
wt/wt
wt/wt
wt/erbB2
erbB2/erbB2
erbB2/erbB2
Y1028F/Y1028F
Y1028F/Y1028F
Y1028F/Y1028F
erbB2
pgk
1 2 3 4 5 6 7 8

protection assay (Figure 4D). The results showed that samples harboring either the *erbB2* or the *Y1028F* knock-in allele expressed identical levels of *erbB2* transcripts (Figure 4D, lanes 4-5 vs. 6-8). Note that either knock-in alleles express significantly lower levels of *erbB2* transcripts compared to wild type animals. This phenomenon is likely a result of using a cDNA knock-in strategy and explains the overall lower ErbB2 expression levels in the knock-in animals. Regardless, our observations argue that specifically expressing the *Y1028F* knock-in allele results in increased ErbB2 protein levels without affecting transcriptional activity, when compared to the *erbB2* knock-in allele.

Y1028 influences ErbB2 receptor turnover rate

Since many receptor tyrosine kinases are downregulated by endocytosis and subsequently targeted for degradation (Katzmann et al., 2002), we examined whether Y1028 affected ErbB2 receptor turnover rate. Rat-1 cell lines stably expressing oncogenic ErbB2 (V664E mutation) or its mutant tyrosine phosphorylation site derivatives were established. Specifically, we compared the turnover rate of the oncogenic ErbB2 receptor versus the Y1028F mutant receptor (Figure 5A-B). Conversely, we also assessed the effects of restoring Y1028 to the Y1144 add-back mutant to generate the Y1028/Y1144 double add-back mutant ErbB2 receptor (Figure 5C-D). Add-back mutants are derived from an ErbB2 receptor stripped of the five major tyrosine autophosphorylation sites by tyrosine-to-phenylalanine mutations and then individual mutant sites are 'added-back' or reverted to tyrosine residues.

Pulse-chase analyses using Rat-1 derived stable cell lines revealed that the Y1028F mutation significantly stabilized ErbB2 compared to the wild type receptor (Figure 5A-B). Alternatively, when Y1028 was restored to the Y1144 add-back mutant, the receptor turnover rate of ErbB2-Y1028/Y1144 increased (Figure 5C-D). Endocytosis and turnover of ErbB2 receptor at the plasma membrane, as determined by monitoring biotin-labeled surface receptor (data not shown), was consistent with the ^{35}S pulse-chase data. Based on these results, we next examined whether the E3-ubiquitin ligase, c-Cbl, was responsible for mediating the negative regulatory effect of Y1028 on ErbB2 activity since c-Cbl has been shown to mediate the downregulation of the EGFR and other receptor tyrosine kinases (Thien and Langdon, 2001). Our data suggest that c-Cbl is able to associate with ErbB2 through other specific phosphotyrosine residues and that Y1028 is not responsible for the recruitment of c-Cbl to ErbB2, nor is it required for the ubiquitylation of ErbB2 (Figure 5E). Taken together, these observations suggest that Y1028 modulates ErbB2 protein levels through a c-Cbl and ubiquitylation independent manner.

Differential ErbB2 signaling requirements in development

Although knock-in animals expressing a single ErbB2 receptor tyrosine phosphorylation site mutation were grossly normal, however, it was conceivable that there may be subtle defects as a result of ablating a particular ErbB2 initiated signaling pathway. Thus, whole embryos (E12.5) were immunostained with anti-neurofilament (Figure 6) to examine the appearance of sensory cutaneous nerves, which were

Figure 5. Tyrosine 1028 promotes the downregulation of ErbB2 in Rat-1 cells.

The stability of mutant ErbB2 receptors in the presence or absence of Y1028 was examined by pulse-chase analyses using ^{35}S-methionine labeled Rat-1 stable cell lines expressing the constitutively active oncogenic ErbB2 mutants. (**A**) ErbB2 versus ErbB2-Y1028F. (**B**) ErbB2-Y1144 add-back mutant versus ErbB2-Y1028/Y1144 double add-back mutant. Representative gels are shown for each and the average of multiple experiments is depicted graphically as a percentage of the original ErbB2 levels remaining.

(**C**) The ubiquitylation status of ErbB2 and its tyrosine phosphorylation mutants expressed transiently in 293T cells were examined by immunoprecipitation using an anti-ErbB2 antibody and then blotting the top half of the membrane with an anti-ubiquitin antibody (Santa Cruz). The blots were then stripped and probed with an anti-ErbB2 antibody to check for equal levels of ErbB2. The bottom half of the membrane was incubated with anti-Cbl antibodies (Santa Cruz) to examine ErbB2-Cbl interactions. The phosphorylation status of c-Cbl when co-expressed with different ErbB2 mutants was examined by immunoprecipitation of c-Cbl followed by blotting with an anti-phosphotyrosine antibody (Transduction Labs). ErbB2* = constitutively activated (oncogenic) ErbB2; Y1028F is the point mutation; NYPD is the tyrosine deficient ErbB2 mutant; Y1028 is the Y1028 add-back mutant.

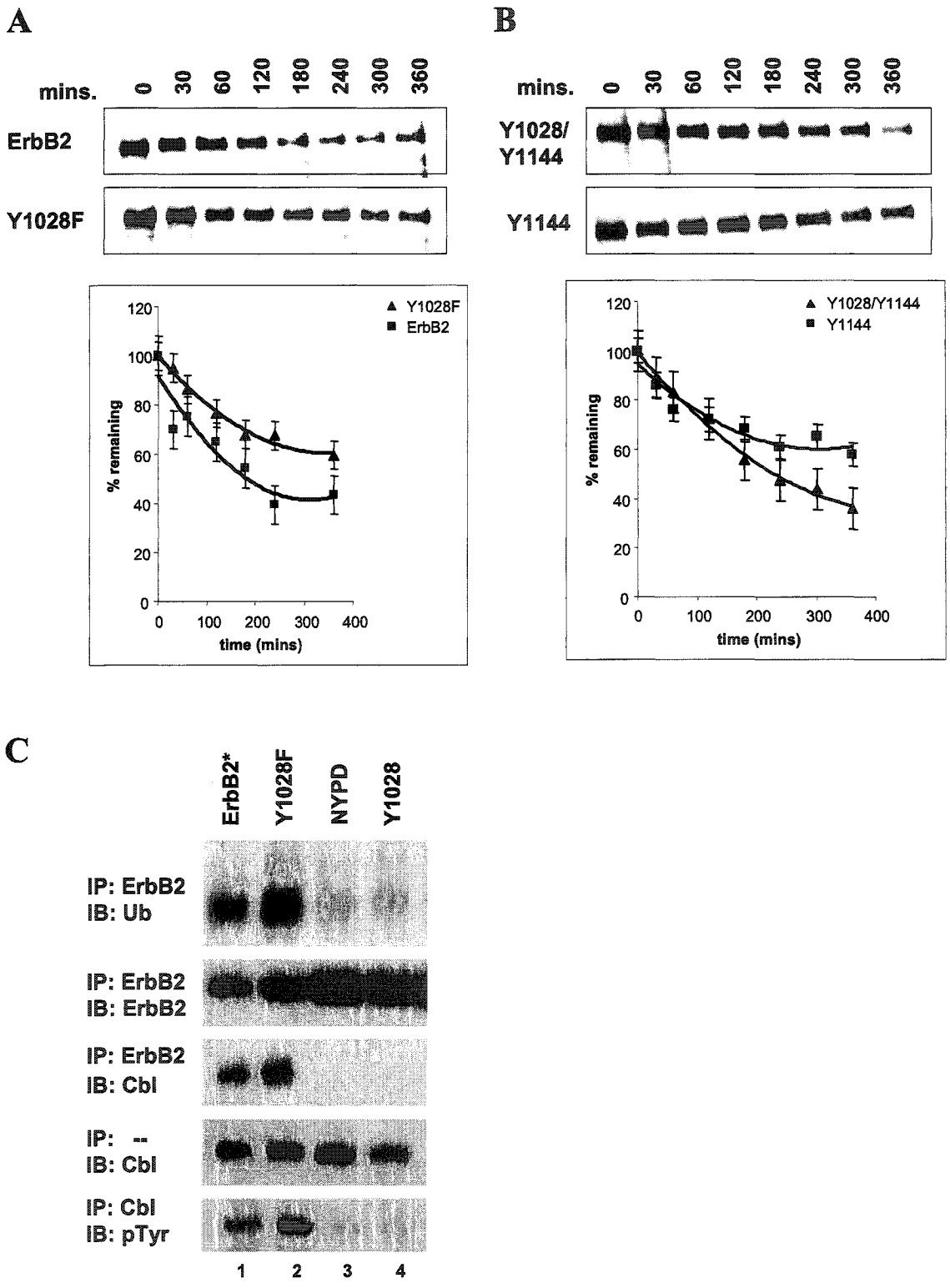
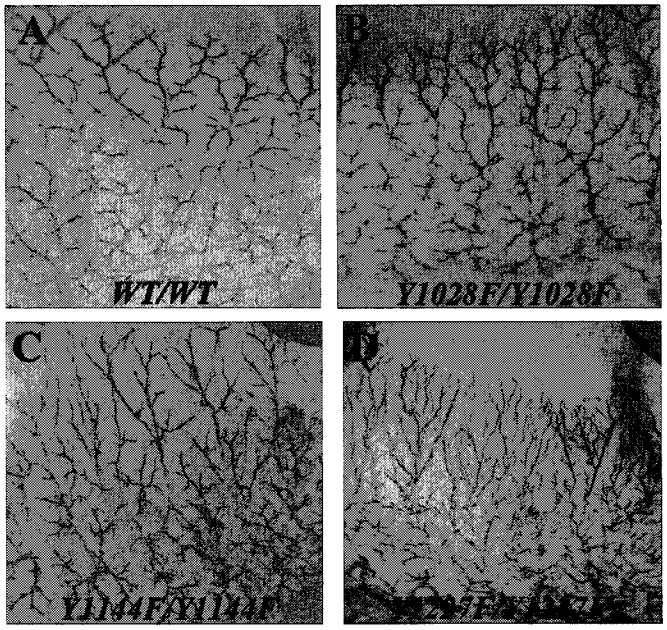

A
mins.
0
30
60
120
180
240
300
360
ErbB2
Y1028F
Y1028F
ErbB2
% remaining
time (mins)
B
mins.
Y1028/
Y1144
Y1144
Y1028/Y1144
Y1144
% remaining
time (mins)
C
ErbB2*
Y1028F
NYPD
Y1028
IP: ErbB2
IB: Ub
IP: ErbB2
IB: ErbB2
IP: ErbB2
IB: Cbl
IP: --
IB: Cbl
IP: Cbl
IB: pTyr
1
2
3
4

Figure 6. Sensory nerve defects in Y1227F knock-in mutants.

Homozygous E12.5 knock-in embryos were subjected to whole-mount immunostaining using anti-neurofilament antibodies. The morphologic appearance of the sensory cutaneous nerves in the thoracic cavity wall region is shown for (**A**) $erbB2^{wt/wt}$ (**B**) $erbB2^{Y1028F/Y1028F}$ (**C**) $erbB2^{Y1144F/Y1144F}$ and (**D**) $erbB2^{Y1227F/Y1227F}$. Note that the nerves in (**D**) are disorganized and defasciculated. (Original magnification: 4x)


A
WT/WT
B
Y1028F/Y1028F
C
Y1144F/Y1144F

abnormally defasciculated in the cardiac rescued *erbB2* mutants and in *erbB3* deficient mutants (Woldeyesus et al., 1999). Interestingly, in comparison with $erbB2^{wt/wt}$ embryos (Figure 6A), there was a striking defect in the morphology of the developing cutaneous nerves in the thoracic body wall of $erbB2^{Y1227F/Y1227F}$ embryos (Figure 6D) that was not observed in $erbB2^{Y1028F/Y1028F}$ or $erbB2^{Y1144F/Y1144F}$ knock-in embryos (Figures 6B, C). The trajectories of the sensory nerves in $erbB2^{Y1227F/Y1227F}$ mutants appeared intact but were highly disorganized and severely defasciculated, similar to the defects in the cardiac rescued *erbB2* mutants (Woldeyesus et al., 1999). These results suggest that the signal transduction pathways initiated specifically by ErbB2-Y1227 is absolutely required for the proper development of the cutaneous sensory nerves in the thoracic body wall.

Discussion

Using a series of *erbB2* cDNA knock-in animals encoding ErbB2 receptors with different tyrosine autophosphorylation site mutations, we have identified a minimum threshold level of ErbB2 (above ~5% of wild type levels) that is required to sustain viability and physiological function. We have also presented evidence suggesting that the individual ErbB2 tyrosine phosphorylation sites have specific and unique roles in development. Inadvertently, the cDNA knock-in allele turned out to be a hypomorph expressing only ~10% the level of ErbB2 expressed in wild type littermates. Despite the low levels of ErbB2, homozygous knock-in animals were viable and overtly normal and healthy. However, when the *erbB2* knock-in animals were intercrossed with *erbB2* deficient animals to generate hemizygous knock-in animals ($erbB2^{erbB2/ko}$), this additional

2-fold reduction in ErbB2 levels fell below the threshold level and resulted in perinatal lethality due to acute respiratory distress. The inability of newborn *erbB2*$^{erbB2/ko}$ pups to inflate their lungs is remarkably similar in phenotype to the cardiac specific rescue of *erbB2* deficient mutants (Morris et al., 1999; Woldeyesus et al., 1999).

Evaluation of the diaphragm muscles of E18.5 embryos revealed an obvious defect in the phrenic nerve of mutant embryos. In the cardiac rescued *erbB2* mutants, Woldeyesus et al. (1999) reported that the phrenic nerve was thin and poorly fasciculated at E14.5 but only remnants were detected at later stages. Observations by Morris et al. (1999) showed that diaphragms of E18.5 mutant embryos were completely devoid of innervations and AChR clusters were more dispersed and rounded in shape. Interestingly, the diaphragm muscles of our *erbB2*$^{erbB2/ko}$ embryos at E18.5 (Figure 2) were innervated, albeit there lacked a main nerve trunk and any nerves that were present were very thin, highly disorganized and fragmented. We also did not observe any differences in the density or shape of AChR receptor clusters compared to wild type embryos. These results support the notion of progressive threshold sensitivities to the level of ErbB2 signaling in the developing peripheral nervous system. And although the low levels of ErbB2 prevented or impeded the complete degeneration of motor neurons in the diaphragm muscle and maintained apparently normal clustering of AChR at the neuromuscular junctions, this was not sufficient for the diaphragm to respond and function. Also note that the low levels of ErbB2 was sufficient to bypass the cardiac trabeculation defects in *erbB2* null embryos, suggesting that cardiac system is not as sensitive as the peripheral nervous system to this threshold level of ErbB2.

Conversely, we were able to genetically rescue the perinatal lethality in hemizygous *erbB2*$^{erbB2/ko}$ embryos by similarly introducing the *Y1028F* knock-in allele into an *erbB2* deficient background to generate *erbB2*$^{Y1028F2/ko}$ animals. Tyrosine 1028 is a negative regulatory signal that affects the stability of the receptor (discussed below). All ErbB2 knock-in mice bearing the *Y1028F* allele expressed higher levels of ErbB2 protein than in the *erbB2* knock-in mice. Thus, this genetic manipulation ablating a negative regulatory phosphotyrosine site in the carboxyl-terminus increased the level of ErbB2 above the minimal threshold required for the phrenic nerve in the diaphragm to develop normally. *ErbB2*$^{Y1028F2/ko}$ animals survived and were phenotypically healthy. In contrast, similar experiments performed with the *Y1144F* or the *Y1227F* knock-in alleles also resulted in perinatal lethality with similar defects in the innervation of the diaphragm muscle. These two mutations, of course, did not affect the level of ErbB2 expressed in the knock-in animals.

Although previous *in vitro* experiments concluded that Y1028 negatively regulated oncogenic ErbB2-mediated transformation, we were also interested in determining if Y1028 would also have this suppressive effect *in vivo* in the context of a non-oncogenic ErbB2 receptor. As described above, the results of the *in vivo* experiments with the Y1028F knock-in animals are consistent with a negative regulatory effect of Y1028 on ErbB2 activity. Moreover, here we have also identified the biochemical basis for this effect, which was previously unknown. Phosphotyrosine 1028 modulates ErbB2 protein levels by promoting the downregulation and turnover of the receptor in a c-Cbl and ubiquitin independent manner. Conversely, loss of Y1028

resulted in an increase in ErbB2 protein stability. Thus, the stabilization of ErbB2 levels above the minimum threshold in the hemizygous *erbB2*$^{Y1028F2/ko}$ animals allowed these particular strains to develop normally.

Sequence alignment of ErbB2 with the EGFR shows that ErbB2-Y1028 and its surrounding sequences share a high degree of similarity to the region surrounding the EGFR-Y992 (Figure 1D). Despite the identity and function of EGFR-Y992 in 1989 (Chen et al., 1989), the mechanism of its function has not been clearly elucidated. Carboxyl-terminal truncation of the EGFR has suggested that an 18 amino acid region surrounding Y992 conforms to an "internalization" domain and is required for EGF-dependent receptor internalization. However, a subsequent study showed that point mutation of Y992 did not affect EGFR internalization (Sorkin et al., 1992). In another study, the Y992F mutation actually increased the rate of EGFR internalization and they suggested that the increase in negative charge associated with phosphorylation of Y992 would reduce the rate of ligand-induced endocytosis (Holbrook et al., 1999). The nature of these discrepancies may be due to artefactual differences in cell types, the level of ectopic EGFR expression in the cells, or the nature of the mutation used in their analyses. Our results showing the effect of Y1028, in a physiologically relevant context, to promote the turnover rate of ErbB2 receptor from the cell surface should lead to clarification of these conflicting reports concerning the role of Y992 in the EGFR.

Homozygous knock-in animals expressing ErbB2 phosphotyrosine mutations where the Grb2 site (Y1144) or the Shc binding site (Y1227) were ablated had little impact on the gross development of mice, even at 10-fold less expression. However,

previous studies in our lab realized a significant reduction in the number of muscle spindle cells in *erbB2*$^{erbB2/erbB2}$ animals, without any obvious phenotypic consequences (Andrechek et al., 2002). Therefore, it is conceivable that other specific cell or tissue types may be more sensitive to the altered ErbB2 signaling. Indeed, the cutaneous sensory nerves in the thoracic body wall of *erbB2*$^{Y1227F/1227F}$ mutants were thin and defasciculated, whereas *erbB2*$^{Y1144F/Y1144F}$ embryos were similar to wild type.

Since ErbB2-Y1227 binds to the Shc adapter protein, these results suggest that the Shc signaling pathway may play an important and unique role downstream of ErbB2 in the development of these sensory nerves. Furthermore, it is interesting to note that Shc can recruit phosphatidylinositol 3' kinase (PI3'K) (Gu et al., 2000). PI3'K is a major signaling pathway downstream of ErbB3, which also display a strikingly similar abnormal sensory nerve phenotype in *erbB3* deficient animals (Woldeyesus et al., 1999). Thus, it is conceivable that the ErbB2 and ErbB3 signaling pathways converge downstream at the level of PI3'K, such that PI3'K plays a key role in the development of sensory nerves. In general, this raises the idea that signaling pathways downstream of the individual receptor phosphotyrosine sites can function differentially and independently in the development of specific tissues.

Unlike ErbB2 where loss of the Grb2 binding site (Y1144) did not result in any discernable phenotype, uncoupling of Grb2 from the Met receptor in a knock-in model resulted in severe defects in muscle development (Maina et al., 1996). Taken together, these observations suggest that the remaining ErbB2 autophosphorylation sites are able to functionally substitute for the inability of these ErbB2 mutants to recruit

Grb2. Alternatively, it is possible that the ErbB2 heterodimerization partners such as EGFR, ErbB3 or ErbB4 can compensate for the lack of Grb2 binding sites on ErbB2. Indeed, it has been demonstrated that EGFR or ErbB3 can independently bind Shc and Grb2 (Batzer et al., 1994; Carraway and Cantley, 1994; Okutani et al., 1994; Prigent and Gullick, 1994). It is also possible that there may be other more subtle developmental defects in different tissues of the *erbB2*^{Y1144F} knock-in mutants. In this regard, transgenic mice expressing constitutively activated ErbB2 mutants coupled to either Grb2 or Shc specifically in the mammary epithelium develop morphologically distinct mammary tumors that possess inherently different metastatic properties (Dankort et al., 2001a).

In summary, using a series of unique *erbB2* knock-in animals and genetic manipulation, we have established that a minimum threshold level of ErbB2 receptor expression is required during proper development. This critical threshold is only 5-10% of normal ErbB2 levels in wild type embryos. We have also identified an important *in vivo* function of the intrinsic negative regulatory site in ErbB2 (Y1028) to modulate the stability/turnover rate of the receptor throughout development. In addition, our data suggests that individual ErbB2 signaling pathways are not redundant but rather play unique roles in specific tissues throughout development.

Experimental Procedures

Knock-in animals

cDNAs encoding the rat ErbB2 (rErbB2) receptor harboring single tyrosine phosphorylation mutations were generated from the corresponding C-terminal domain of the oncogenic ErbB2 mutants previously described by Dankort et al. (Dankort et al., 1997). The cDNAs were cloned into the targeting vector containing flanking 3' and 5' arms of homology as described previously (Chan et al., 2002). These plasmids were electroporated into R1 ES cells and G418 (Geneticin; GIBCO) resistant colonies were picked and subsequently screened by Southern Blot analysis for correctly targeted mutants. Mutant mice were generated from the positive ES cell clones and were maintained in an SV129/Balb/c background.

Immunoblot Analysis of Embryo Lysates

Embryos were lysed in modified TNE lysis buffer (50mM Tris- HCl pH 7.6, 150mM NaCl, 1% NP-40, 10mM NaF, 2mM EDTA) supplemented with 10μg/ml leupeptin, 10μg/ml aprotinin and 1mM sodium orthovanadate. For immunoblotting, the membranes were incubated with an anti-ErbB2 monoclonal (Ab-3, Oncogene Research) or anti-Grb2 polyclonal (1:2500; C-23, Santa Cruz) antibody overnight at 4°C. Subsequently, the membranes were treated with anti-mouseHRP or anti-rabbitHRP secondary antibodies for 1 hour at room temperature, washed three times in TBS and visualized by enhanced chemiluminescence (Amersham), as specified by the manufacturer.

Whole-mount in situ

For whole-mount *in situ* hybridizations, embryos were fixed in 4% paraformaldehyde/0.2% glutaraldehyde, dehydrated through a graded series of methanol/PBT (PBS + 0.1% Tween-20) baths and stored in 100% MeOH at -20°C until needed. Whole-mount *in situ* hybridizations were carried out as previously described (Wilkinson and Nieto, 1993).

Immunohistochemistry

E12.5 embryos were fixed in fresh 4% paraformaldehyde, washed several times with PBS and then post-fixed and bleached in Dent's fixative (methanol:DMSO, 4:1) + 5% H_2O_2. After extensive washes, the embryos were incubated in blocking solution (5% goat serum + 1% DMSO in TBS) overnight at room temperature. Incubation in anti-NF150 (1:1000, Chemicon) diluted in TBS + 1% DMSO was performed for 2 days at room temperature. After 5 x 1 hour washes in TBS-T (TBS + 1% Tween20), the embryos were incubated overnight in goat anti-rabbitHRP (1:1000 in TBS-T). DAB+ kit (DAKO, cat# K3468) was used to visualize the neurofilament staining. Whole-mount immunohistochemistry was performed on diaphragm dissected from E18.5 embryos and fixed in 4% PFA. They were first treated in 0.1M Glycine in PBS, washed several times, then incubated in PBT (PBS + 0.5% Triton-X-100) for 5 minutes. Diaphragms were incubated overnight with anti-NF150 diluted 1:1000 in PBT + 5% Goat Serum. After extensive washes in PBT, they were incubated overnight with goat anti-rabbit-Alexa488 (Molecular Probes) diluted 1:1000 in PBT and α-bungarotoxin-Alexa594 (Molecular

Probes). Diaphragms were visualized using the Zeiss LSM 510Meta confocal microscope.

Receptor turnover assay

Rat-1 cells stably expressing the oncogenic ErbB2 tyrosine phosphorylation site mutants were seeded (1 x 10^6 cells) onto 60mm plates and pulse labeled using 0.1 mCi/ml of ^{35}S-EXPRESS Protein Labeling mix (NEG772, NEN Life Science Products). The cells were then washed and chased with unlabelled medium for the indicated times. Protein lysates from the labeled cells were immunoprecipitated using an anti-ErbB2 antibody (Ab4, Oncogene Science) and resolved on an 8% SDS-PAGE. The gel was dried and exposed to a PhosphoImager screen (Kodak) and scanned using the Typhoon8600 scanner (Amersham). Image analyses were performed using ImageQuant software (Molecular Dynamics).

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References

Akiyama, T., Matsuda, S., Namba, Y., Saito, T., Toyoshima, K., and Yamamoto, T. (1991). The transforming potential of the c-erbB-2 protein is regulated by its autophosphorylation at the carboxyl-terminal domain. Mol Cell Biol *11*, 833-842.

Andrechek, E. R., Hardy, W. R., Girgis-Gabardo, A. A., Perry, R. L., Butler, R., Graham, F. L., Kahn, R. C., Rudnicki, M. A., and Muller, W. J. (2002). ErbB2 is required for muscle spindle and myoblast cell survival. Mol Cell Biol *22*, 4714-4722.

Batzer, A. G., Rotin, D., Urena, J. M., Skolnik, E. Y., and Schlessinger, J. (1994). Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. Mol Cell Biol *14*, 5192-5201.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C., and Riethmacher, D. (1998). The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. Genes Dev *12*, 1825-1836.

Carraway, K. L., 3rd, and Cantley, L. C. (1994). A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. Cell *78*, 5-8.

Carraway, K. L., 3rd, Rossi, E. A., Komatsu, M., Price-Schiavi, S. A., Huang, D., Guy, P. M., Carvajal, M. E., Fregien, N., Carraway, C. A., and Carraway, K. L. (1999). An intramembrane modulator of the ErbB2 receptor tyrosine kinase that potentiates neuregulin signaling. J Biol Chem *274*, 5263-5266.

Carraway, K. L., 3rd, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C., and Cerione, R. A. (1994). The erbB3 gene product is a receptor for heregulin. J Biol Chem *269*, 14303-14306.

Chan, R., Hardy, W. R., Laing, M. A., Hardy, S. E., and Muller, W. J. (2002). The catalytic activity of the ErbB-2 receptor tyrosine kinase is essential for embryonic development. Mol Cell Biol *22*, 1073-1078.

Chen, W. S., Lazar, C. S., Lund, K. A., Welsh, J. B., Chang, C. P., Walton, G. M., Der, C. J., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1989). Functional

independence of the epidermal growth factor receptor from a domain required for ligand-induced internalization and calcium regulation. Cell *59*, 33-43.

Dankort, D., Jeyabalan, N., Jones, N., Dumont, D. J., and Muller, W. J. (2001). Multiple ErbB-2/Neu Phosphorylation Sites Mediate Transformation through Distinct Effector Proteins. J Biol Chem *276*, 38921-38928.

Dankort, D. L., Wang, Z., Blackmore, V., Moran, M. F., and Muller, W. J. (1997). Distinct tyrosine autophosphorylation sites negatively and positively modulate neu-mediated transformation. Mol Cell Biol *17*, 5410-5425.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R., and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature *378*, 390-394.

Goldman, R., Levy, R. B., Peles, E., and Yarden, Y. (1990). Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. Biochemistry *29*, 11024-11028.

Gu, H., Maeda, H., Moon, J. J., Lord, J. D., Yoakim, M., Nelson, B. H., and Neel, B. G. (2000). New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. Mol Cell Biol *20*, 7109-7120.

Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L., 3rd (1994). Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc Natl Acad Sci U S A *91*, 8132-8136.

Hazan, R., Margolis, B., Dombalagian, M., Ullrich, A., Zilberstein, A., and Schlessinger, J. (1990). Identification of autophosphorylation sites of HER2/neu. Cell Growth Differ *1*, 3-7.

Holbrook, M. R., O'Donnell, J. B., Jr., Slakey, L. L., and Gross, D. J. (1999). Epidermal growth factor receptor internalization rate is regulated by negative charges near the SH2 binding site Tyr992. Biochemistry *38*, 9348-9356.

Hynes, N. E., and Stern, D. F. (1994). The biology of erbB-2/neu/HER-2 and its role in cancer. Biochim Biophys Acta *1198*, 165-184.

Karunagaran, D., Tzahar, E., Beerli, R. R., Chen, X., Graus-Porta, D., Ratzkin, B. J., Seger, R., Hynes, N. E., and Yarden, Y. (1996). ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. Embo J *15*, 254-264.

Katzmann, D. J., Odorizzi, G., and Emr, S. D. (2002). Receptor downregulation and multivesicular-body sorting. Nat Rev Mol Cell Biol *3*, 893-905.

Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1995). PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. Science *268*, 1177-1179.

King, C. R., Borrello, I., Bellot, F., Comoglio, P., and Schlessinger, J. (1988). Egf binding to its receptor triggers a rapid tyrosine phosphorylation of the erbB-2 protein in the mammary tumor cell line SK-BR-3. Embo J *7*, 1647-1651.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C., and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature *378*, 394-398.

Maina, F., Casagranda, F., Audero, E., Simeone, A., Comoglio, P. M., Klein, R., and Ponzetto, C. (1996). Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. Cell *87*, 531-542.

Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D., and Lee, K. F. (1999). Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. Neuron *23*, 273-283.

Okutani, T., Okabayashi, Y., Kido, Y., Sugimoto, Y., Sakaguchi, K., Matuoka, K., Takenawa, T., and Kasuga, M. (1994). Grb2/Ash binds directly to tyrosines 1068 and 1086 and indirectly to tyrosine 1148 of activated human epidermal growth factor receptors in intact cells. J Biol Chem *269*, 31310-31314.

Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. Embo J *19*, 3159-3167.

Pawson, T. (1995). Protein modules and signalling networks. Nature *373*, 573-580.

Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D., and Yarden, Y. (1993). Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests complex ligand-receptor relationships. Embo J *12*, 961-971.

Pinkas-Kramarski, R., Lenferink, A. E., Bacus, S. S., Lyass, L., van de Poll, M. L., Klapper, L. N., Tzahar, E., Sela, M., van Zoelen, E. J., and Yarden, Y. (1998a). The oncogenic ErbB-2/ErbB-3 heterodimer is a surrogate receptor of the epidermal growth factor and betacellulin. Oncogene *16*, 1249-1258.

Pinkas-Kramarski, R., Shelly, M., Glathe, S., Ratzkin, B. J., and Yarden, Y. (1996a). Neu differentiation factor/neuregulin isoforms activate distinct receptor combinations. J Biol Chem *271*, 19029-19032.

Pinkas-Kramarski, R., Shelly, M., Guarino, B. C., Wang, L. M., Lyass, L., Alroy, I., Alimandi, M., Kuo, A., Moyer, J. D., Lavi, S., *et al.* (1998b). ErbB tyrosine kinases and the two neuregulin families constitute a ligand-receptor network. Mol Cell Biol *18*, 6090-6101.

Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M., and Yarden, Y. (1996b). Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. Embo J *15*, 2452-2467.

Prigent, S. A., and Gullick, W. J. (1994). Identification of c-erbB-3 binding sites for phosphatidylinositol 3'-kinase and SHC using an EGF receptor/c-erbB-3 chimera. Embo J *13*, 2831-2841.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R., and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature *389*, 725-730.

Simpson, B. J., Phillips, H. A., Lessells, A. M., Langdon, S. P., and Miller, W. R. (1995). c-erbB growth-factor-receptor proteins in ovarian tumours. Int J Cancer *64*, 202-206.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science *235*, 177-182.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science *244*, 707-712.

Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., 3rd (1994). Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J Biol Chem *269*, 14661-14665.

Sorkin, A., Helin, K., Waters, C. M., Carpenter, G., and Beguinot, L. (1992). Multiple autophosphorylation sites of the epidermal growth factor receptor are essential for receptor kinase activity and internalization. Contrasting significance of tyrosine 992 in the native and truncated receptors. J Biol Chem *267*, 8672-8678.

Stern, D. F., and Kamps, M. P. (1988). EGF-stimulated tyrosine phosphorylation of p185neu: a potential model for receptor interactions. Embo J *7*, 995-1001.

Thien, C. B., and Langdon, W. Y. (2001). Cbl: many adaptations to regulate protein tyrosine kinases. Nat Rev Mol Cell Biol *2*, 294-307.

Tzahar, E., Pinkas-Kramarski, R., Moyer, J. D., Klapper, L. N., Alroy, I., Levkowitz, G., Shelly, M., Henis, S., Eisenstein, M., Ratzkin, B. J., *et al.* (1997). Bivalence of EGF-like ligands drives the ErbB signaling network. Embo J *16*, 4938-4950.

Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996). A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. Mol Cell Biol *16*, 5276-5287.

Wilkinson, D. G., and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. Methods Enzymol *225*, 361-373.

Woldeyesus, M. T., Britsch, S., Riethmacher, D., Xu, L., Sonnenberg-Riethmacher, E., Abou-Rebyeh, F., Harvey, R., Caroni, P., and Birchmeier, C. (1999). Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. Genes Dev *13*, 2538-2548.

Yarden, Y., and Sliwkowski, M. X. (2001). Untangling the ErbB signalling network. Nat Rev Mol Cell Biol *2*, 127-137.

Chapter 5

The Tyrosine Kinase Region of ErbB2 Renders the Receptor Refractory to c-Cbl Mediated Ubiquitylation

Preface

To be consistent with the format of the previous two chapters, the work in Chapter 5 also will be presented in a manuscript format. At this time, this manuscript has not been submitted for publication.

In Chapter 4, examination into the mechanism of Y1028-mediated downregulation of ErbB2 also led to the discovery of an ErbB2/c-Cbl association and ubiquitylation of the receptor, which is dependent on the presence of at least one of the tyrosine autophosphorylation sites other than Y1028. To follow up on this, Chapter 5 explores in greater depth the nature of c-Cbl interaction with ErbB2 and its function, and also the ubiquitylation status of ErbB2 is characterized and described. Unlike the work presented in Chapters 3 or 4, this chapter moves into in vitro analyses based on the conclusions from data collected from the in vivo work. However, as will be described in Chapter 5, the results of this investigation regarding c-Cbl association with ErbB2 and ErbB2 ubiquitylation are very intriguing and will surely provide the basis to lead to further examination of these effects, in vivo.

The tyrosine kinase region of ErbB2 renders the receptor refractory to c-Cbl mediated ubiquitylation

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Running Title: **Interaction of ErbB2 and c-Cbl**

Abstract

Many receptor tyrosine kinases are regulated by the catalytic action of the c-Cbl ubiquitin ligase. Loss of c-Cbl binding leads to deregulation of receptor activity and enhanced signaling. Here we describe the association of c-Cbl with ErbB2 at Y1144 and Y1226/7 within the C-terminus of ErbB2. Both of these tyrosine phosphorylation sites also bind to Grb2, either directly or indirectly, which likely mediate the interaction between c-Cbl and ErbB2. Unlike with the EGFR, a wild type c-Cbl protein does not bind directly to ErbB2 via its tyrosine kinase binding domain. We also demonstrate that ErbB2 is refractory to c-Cbl mediated ubiquitylation and downregulation since their association does not correspond to ErbB2 ubiquitylation. Furthermore, we identify the tyrosine kinase domain within the ErbB2 receptor as a major factor in determining the receptor's sensitivity to c-Cbl. The ubiquitylation of ErbB2 is however dependent on the presence of Y1253, which leads to the monoubiquitylation of ErbB2. Again, the monoubiquitylation of ErbB2 did not lead to attenuation or lysosomal degradation of the receptor.

Introduction

The ErbB2/Neu receptor tyrosine kinase belongs to the Epidermal Growth Factor Receptor (EGFR) family, which also includes the EGFR, ErbB3, and ErbB4. They all share structural similarities including an extracellular ligand binding domain harboring two cysteine-rich regions, a single pass transmembrane domain, a conserved tyrosine kinase domain, and a regulatory carboxy-terminal tail containing several tyrosine autophosphorylation sites (Hynes and Stern, 1994). Ligand stimulation results in strong mitogenic signals emanating from the receptors leading to biological responses including cellular growth, differentiation and survival. As an illustration of their importance, members of the EGFR family are collectively involved in both development and in disease (Burden and Yarden, 1997). For example, whereas amplification and aberrant overexpression of ErbB2 has been implicated in human breast cancer (Slamon et al., 1987; Slamon et al., 1989), the loss of *erbB2* expression in gene targeting studies revealed deleterious effects on the developing embryo where they die at midgestation due to cardiac ventricular defects (Lee et al., 1995).

Following activation of the receptor and receptor homodimerization and/or heterodimerization with other family members, specific tyrosyl residues residing in the carboxy-terminal tail of the receptor become phosphorylated (Akiyama et al., 1991; Hazan et al., 1990). Specific phosphotyrosine sites provide potential binding targets for intracellular signaling proteins harboring phosphotyrosine binding (PTB) (Kavanaugh et al., 1995) or Src homology 2 (SH2) domains (Pawson, 1995). These include, but are not limited to, phospholipase-Cγ (Fazioli et al., 1991; Peles et al., 1991), phosphatidylinositol

3-kinase (PI3'K) (Peles et al., 1992b; Scott et al., 1991), c-Src (Luttrell et al., 1994; Muthuswamy et al., 1994), Grb2 (Dankort et al., 1997; Janes et al., 1994), Grb7 (Stein et al., 1994), Shc (Dankort et al., 1997; Segatto et al., 1993), and RasGAP (Fazioli et al., 1991; Jallal et al., 1992). For the ErbB2 receptor, there are five such major tyrosine autophosphorylation sites (Y1028, Y1144, Y1201, Y1226/7, Y1253) in the carboxy-terminal regulatory region as described by Dankort et al. (1997). Specifically, they identified Y1144 as the direct binding site for Grb2 and Y1226/7 can recruit the Shc adapter protein, which is also bound to Grb2. In addition, it was determined that each of the four sites, other than Y1028, can signal through the Ras-MAPK pathway.

Whereas individual tyrosine-to-phenylalanine mutations at four of the five sites within ErbB2 modestly reduced the in vitro transforming ability of ErbB2, simultaneous mutation of all five sites to phenylalanine to generate the tyrosine phosphorylation deficient mutant (NYPD) drastically impeded transformation of fibroblasts (Dankort et al., 1997). The one remaining autophosphorylation site (Y1028), when mutated, resulted in a consistent increase in cellular transformation. Indeed, ErbB2-Y1028 was recently demonstrated, in vivo, to be a bona fide negative regulator of ErbB2 protein level by promoting its turnover (Chan et al., submitted, 2004). Although this was suspicious of a c-Cbl ubiquitin ligase mediated event leading to receptor downregulation, biochemical analyses proved otherwise since c-Cbl did associate with ErbB2 but not specifically with tyrosine 1028. Consequently, it inspired this current study to examine the nature of the ErbB2/c-Cbl interaction.

Once receptor tyrosine kinases are activated, it is imperative to regulate their activity to ensure proper signaling intensity for normal growth and development. It is well established now that the protooncogene c-Cbl can function as a negative regulator of RTKs through its E3 ubiquitin ligase activity (Joazeiro et al., 1999; Levkowitz et al., 1999; Thien and Langdon, 2001). The c-Cbl protein contains an N-terminal tyrosine kinase binding (TKB) domain containing a variant SH2 domain that recognizes phosphotyrosine residues (Meng et al., 1999), a RING finger domain that recruits the E2-ubiquitin conjugating enzymes (Zheng et al., 2000), and a C-terminal portion containing a proline-rich stretch and several potential tyrosine phosphorylation sites that may interact with a number of intracellular proteins including Grb2, Nck, Fyn, Vav, Crk and PI3'K (Thien and Langdon). Both the TKB and RING finger domains are necessary and required for c-Cbl targeting and ligand-induced ubiquitylation of the receptor (Lill et al., 2000; Miyake et al., 1999; Thien et al., 2001; Waterman et al., 1999). Alternatively, the c-Cbl proline-rich region can bind to the SH3 domain of the Grb2 adapter protein, which is recruited to a number of RTKs thus providing a surrogate method to couple c-Cbl with its target receptor (Fukazawa et al., 1996; Meisner and Czech, 1995).

Several RTKs are known to interact with and are downregulated by c-Cbl. These include the EGFR, CSF-1R, PDGFR, Met, and Ron receptor tyrosine kinases (Lee et al., 1999; Levkowitz et al., 1998; Miyake et al., 1999; Penengo et al., 2003; Peschard et al., 2001). c-Cbl's enzymatic activity tags RTK substrates with ubiquitin moieties, which directs RTK internalization and endosomal trafficking, ultimately leading to the delivery of the cargo to a lysosomal compartment for degradation (Hicke, 2001; Weissman, 2001).

Recently, it was determined that a single ubiquitin molecule contains the internalization signal and when appended to an activated receptor, it was sufficient to promote the internalization of the receptor from the plasma membrane (Nakatsu et al., 2000; Shih et al., 2000). Although currently it has only been demonstrated for the EGFR and PDGFR (Haglund et al., 2003; Mosesson et al., 2003), it is likely that all RTKs undergoing c-Cbl mediated lysosomal degradation are monoubiquitylated by c-Cbl, rather than polyubiquitylated, which would target the proteins for proteosomal degradation.

In this report, we identify the tyrosine phosphorylation sites in ErbB2 that are required for its association with c-Cbl. Our results suggest that the primary mode of interaction is dependent on either of the two autophosphorylation sites, Y1144 and Y1226/7, which are capable of recruiting the Grb2 adapter protein, either directly or indirectly. However, unlike the EGFR, the association of c-Cbl with ErbB2 did not result in the ubiquitylation of the receptor. We present data to suggest that the difference between the EGFR and ErbB2 in this respect lies in the tyrosine kinase region and its ability to recruit the c-Src protein kinase. Since ErbB2, but not the EGFR, can directly associate with c-Src, it is this difference that protects ErbB2 from the c-Cbl mediated downregulation. We also determined that Y1253 is required for the ubiquitylation of ErbB2 and that it is indeed monoubiquitylated, rather than polyubiquitylated, but unlike other RTKs this did not lead to receptor downregulation.

Materials and Methods

Plasmids and Transfections

cDNAs encoding the constitutively activated V664E (Bargmann et al., 1986a) mutant ErbB2/Neu receptor (NeuNT) or its derivatives harboring tyrosine phosphorylation site mutations were generated previously in our lab (Dankort et al., 1997). The ErbB2-Y1117F mutation was engineered by Harold Kim (unpublished). All ErbB2 cDNAs were subsequently cloned into the pcDNA3 (Invitrogen) expression plasmid. c-Cbl and its mutant derivatives were generous gifts from Morag Park (McGill University). 1 ug of pcDNA3-ErbB2 and 3 ug of pXM-Cbl plasmids were used in transfections of 293T cells using Lipofectamine reagent (Invitrogen) and following the manufacturer's directions.

Cell Culture and Cell Lysates

293T cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum, penicillin and streptomycin (Gibco-BRL). For growth factor stimulation, cells were first serum starved overnight. Stimulation with human Epidermal Growth Factor (Invitrogen) was performed at room temperature for 10 minutes using a concentration of 100 ng/mL. Cells were then washed with phosphate buffered saline and lysed using PLCγ lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% Glycerol, 1% Triton-X-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 10 mM NaF and supplemented with 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM sodium orthovanadate) and cleared by centrifugation at >13000 RPM for 15 minutes at 4°C.

Antibodies, Immunoprecipitation and Immunoblotting

Cleared lysates were immunoprecipitated using anti-ErbB2 antibody (Ab-4, Oncogene Science) and immunoblotting was performed using the following antibodies accordingly: anti-ErbB2 (Ab-3, Oncogene Science; or C18, Santa Cruz), anti-Cbl (C-15, Santa Cruz), anti-Ubiquitin (P4D1, Santa Cruz; or FK1, Affiniti Research), anti-Grb2 (C23, Santa Cruz), anti-HA (HA.11, BABCO/Covance), and anti-c-Src (Ab1, Oncogene Science).

The phosphorylation status of c-Cbl was detected by immunoprecipitation of Cbl using the C-15 antibody and subsequently immunoblotted with anti-phosphotyrosine antibody (PY20; Transduction Labs). The anti-EGFR antibody used for immunoprecipitation and/or immunoblotting was from Transduction Labs (E12020). All primary antibodies used for immunoblotting were diluted 1:1000 in 3% skim milk powder/Tris-Buffered Saline. After the primary antibody incubations, membranes were subjected to 4 x 15 minute washes in TBS-1% Tween-20 (Bio-Rad). Subsequently, horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5000; Jackson Laboratories) were incubated with the membranes for 1 hour at room temperature then washed 2 x 10 minutes in TBS-1% Tween-20 and 2 x 10 minutes in TBS. Immunoblots were visualized by enhanced chemiluminescence (Amersham) as specified by the manufacturer.

Results

Association of c-Cbl with ErbB2-Y1144 and Y1226/7

For clarification, we have used nomenclature that is consistent with the original description of the ErbB2/Neu tyrosine phosphorylation mutants (Dankort et al., 1997). For simplicity, the five tyrosine autophosphorylation sites (Y1028, Y1144, Y1201, Y1226/7, Y1253) in the C-terminus of the receptor were assigned a letter, A through E (Figure 1A). The oncogenic rat *c-neu* is designated NT (for Neu Transforming) and is rendered constitutively active by the V664E mutation in the transmembrane domain (Bargmann et al., 1986a; Weiner et al., 1989b). Single point mutations of the tyrosine residues are termed NT-A, NT-B, NT-C, NT-D, and NT-E, respectively. A mutant variant of this oncogenic receptor where all five tyrosine residues have been simultaneously mutated to phenylalanine was named NYPD (for Neu tyrosine Phosphorylation Deficient). Reversion of individual mutant phosphorylation sites back to tyrosine (the so-called "add-back" mutants) are designated by the following, respectively: YA, YB, YC, YD, and YE.

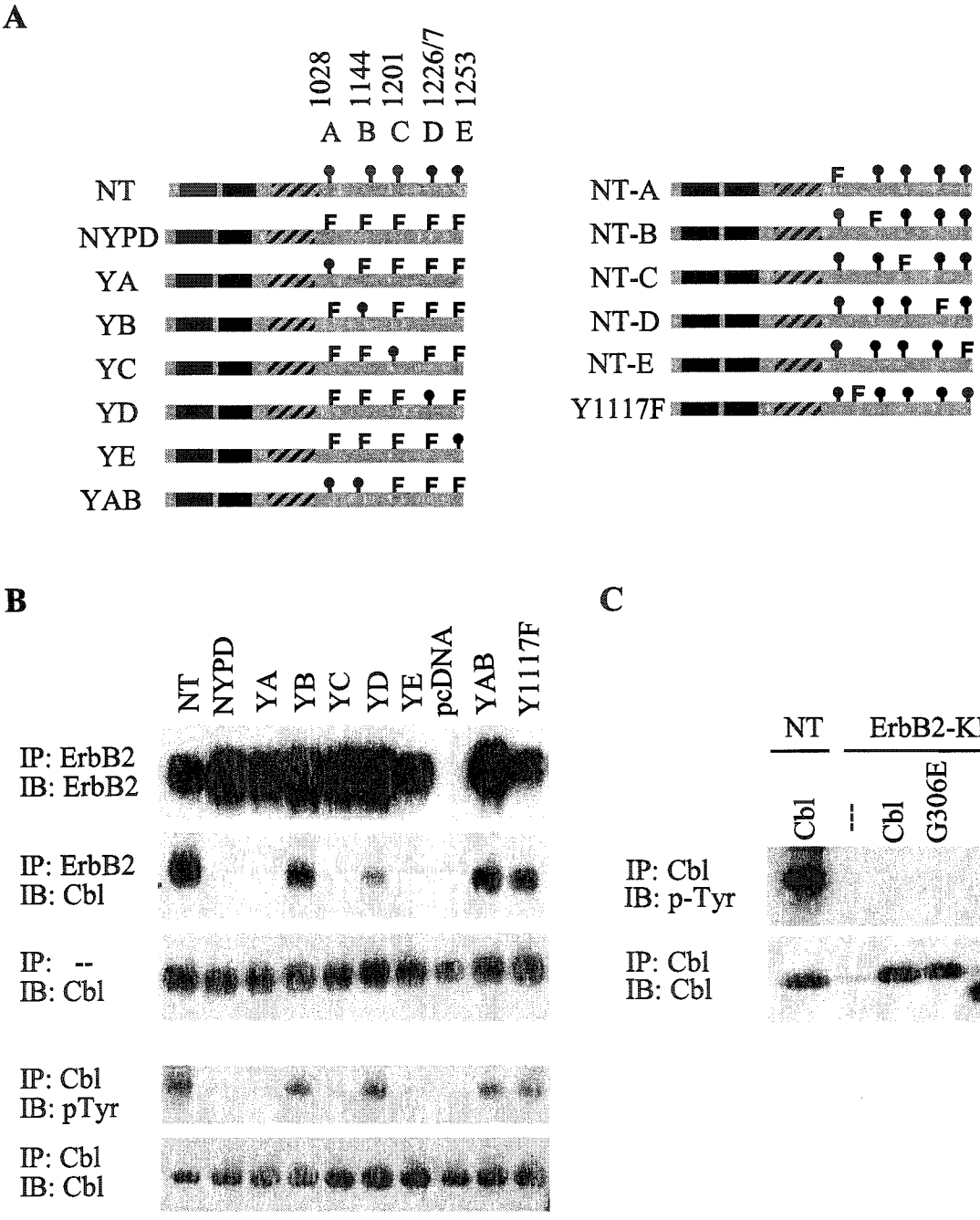
We had previously investigated the negative regulatory effects of ErbB2-Y1028 on ErbB2 signaling and had demonstrated that its actions were independent of c-Cbl mediated ubiquitylation of the receptor. However, we did detect an ErbB2/c-Cbl interaction. Here we identified the tyrosine phosphorylation sites in the C-terminal tail of ErbB2 that is required for the association of c-Cbl to ErbB2 and for efficient ubiquitylation of the receptor.

To examine the interaction of c-Cbl with ErbB2, co-immunoprecipitates were analyzed using lysates prepared from 293T cells co-transfected with plasmids expressing c-Cbl and the different ErbB2 mutants, as indicated (Figure 1B). We were able to co-immunoprecipitate c-Cbl with the ErbB2-NT mutant where all the tyrosine phosphorylation sites are intact. However, this association was completely ablated when the receptor was stripped of the major tyrosine phosphorylation sites (ErbB2-NYPD), suggesting that at least one of the phosphorylation sites is required to couple to c-Cbl. To this end, systematic evaluation of the individual phosphorylation sites revealed that c-Cbl may be recruited to Y1144 (YB) or Y1226/7 (YD) in the C-terminus of ErbB2. Each of these sites alone was sufficient and the presence of either site is necessary for the association of c-Cbl with ErbB2 (Figure 1B). Interestingly, it was previously reported that the Grb2 adapter protein associated with these two sites, either directly (Y1144) or indirectly via the Shc adapter protein (Y1226/7) (Dankort et al., 1997). This was not surprising since it is well documented that there is constitutive association of c-Cbl with Grb2 (Anderson et al., 1997; Buday et al., 1996; Donovan et al., 1996) and several other RTKs, including EGFR, Met, and Ron, are able to recruit c-Cbl in a Grb2 dependent manner (Penengo et al., 2003; Peschard et al., 2001; Waterman et al., 2002).

Consistent with its interaction with the ErbB2 receptor tyrosine kinase, c-Cbl was tyrosine phosphorylated by ErbB2, suggesting that its recruitment to the receptor plays a functional role. Specifically, c-Cbl was tyrosine phosphorylated when co-expressed with constitutively active ErbB2 receptors harboring an intact Y1144 or Y1226/7 (Figure 1B). This phosphorylation of c-Cbl is also dependent on the kinase activity of ErbB2 since

Figure 1. Association of c-Cbl with ErbB2 tyrosine phosphorylation sites.

(A) Schematic diagram of the ErbB2/Neu receptor showing the five major tyrosine autophosphorylation sites in the C-terminus and the corresponding mutants used in this study. NT is the Neu Transforming mutant containing the V664E point mutation in the transmembrane domain. NYPD is the Neu Tyrosine Phosphorylation Deficient mutant. The letters A-E were assigned to represent each autophosphorylation site for simplicity. The numbers indicate the amino acid position for each tyrosine residue. Tyrosine sites that were mutated to phenylalanine are designated by the letter "F". **(B)** The ability of individual ErbB2 tyrosine phosphorylation sites to recruit Cbl was determined by co-transfecting 293T cells with plasmids expressing Cbl and the ErbB2 mutant indicated and performing coimmunoprecipitation assays. Membranes were first incubated with anti-Cbl antibodies, then stripped and re-blotted using anti-ErbB2 antibodies. The phosphorylation status of Cbl was determined by immunoprecipitating Cbl and blotting with anti-phosphotyrosine antibodies. The membranes were then stripped and re-blotted for Cbl to ensure equal levels. **(C)** The phosphorylation status of Cbl was determined when co-expressed with a kinase-defective (KD) ErbB2 receptor. G306E and ΔPro are mutant Cbl proteins.


A
1028
1144
1201
1226/7
1253
A B C D E
NT
NYPD
YA
YB
YC
YD
YE
YAB
NT-A
NT-B
NT-C
NT-D
NT-E
Y1117F
B
NT
NYPD
YA
YB
YC
YD
YE
pcDNA
YAB
Y1117F
IP: ErbB2
IB: ErbB2
IP: ErbB2
IB: Cbl
IP: --
IB: Cbl
IP: Cbl
IB: pTyr
IP: Cbl
IB: Cbl
C
NT
ErbB2-KD
Cbl

Cbl
G306E
ΔPro
IP: Cbl
IB: p-Tyr
IP: Cbl
IB: Cbl

expression of a kinase deficient mutant of ErbB2 (ErbB2-KD) did not result in the concomitant phosphorylation of c-Cbl (Figure 1C).

Full length wild type c-Cbl does not bind to ErbB2-Y1117

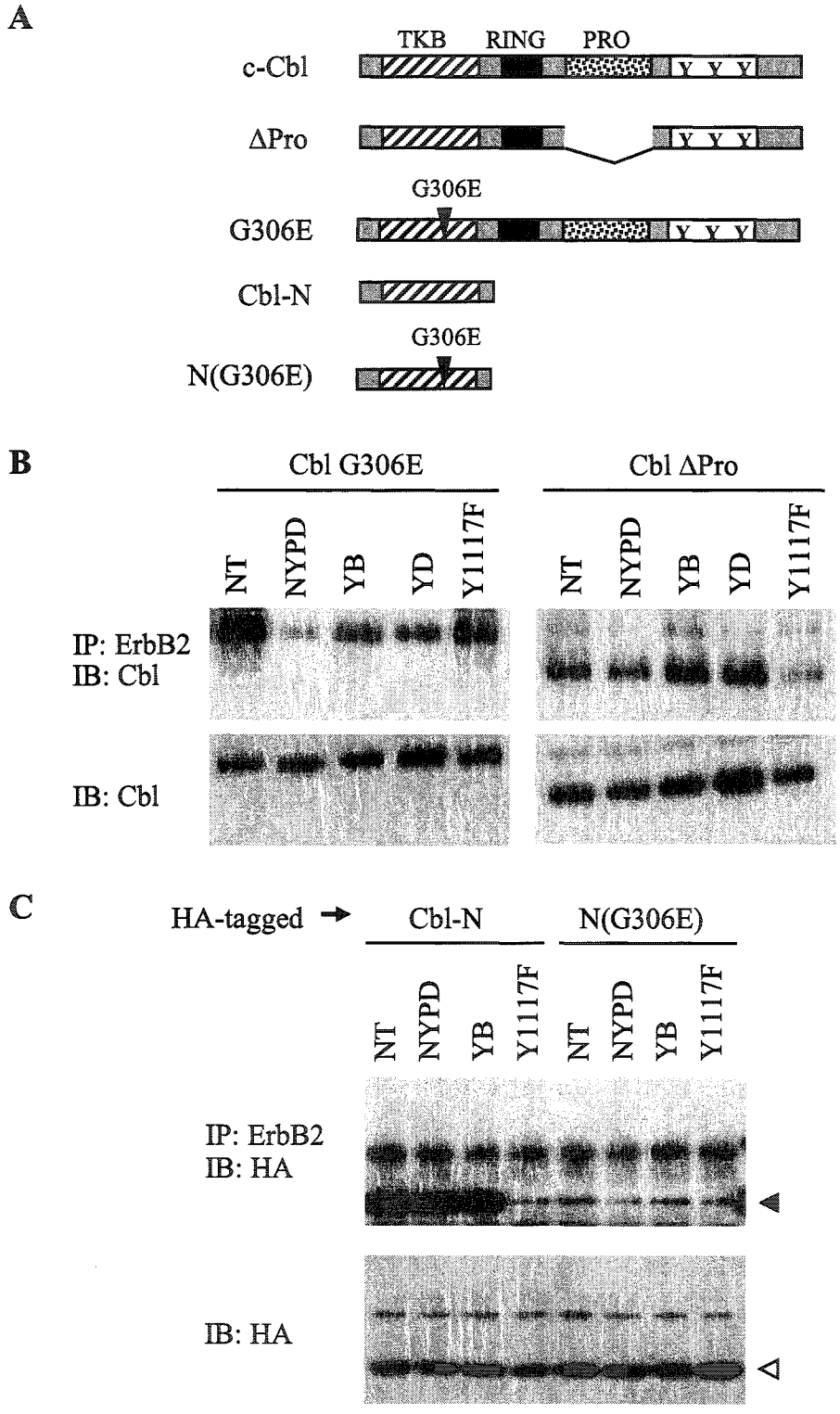
It is interesting to note, however, that c-Cbl was still able to co-immunoprecipitate with an ErbB2 receptor harboring the Y1117F point mutation whereas this interaction was completely ablated with the NYPD mutant where the Y1117 is intact (Figure 1B). This was intriguing because Y1112 in the human ErbB2 receptor (Y1117 in the rat ErbB2 sequence) was previously reported as the direct binding site for c-Cbl via its SH2 containing Tyrosine Kinase Binding (TKB) domain and is required for the ubiquitylation and downregulation of ErbB2 (Klapper et al., 2000). Thus, it was necessary to further characterize the nature of the ErbB2/c-Cbl interaction by using mutant Cbl proteins (Figure 2A) co-expressed with ErbB2 mutants in 293T cells. Loss of the direct binding site in the c-Cbl TKB domain (Cbl-G306E) had no bearing on the ErbB2/Cbl interaction (Figure 2B), which was consistent with the lack of an effect of expressing the ErbB2-Y1117F mutation. This suggested that the c-Cbl TKB region is dispensable for the ErbB2/c-Cbl interaction. The Cbl-G306E mutant behaved similarly as the wild type c-Cbl protein since it also could not associate with the ErbB2-NYPD mutant, which lack the ability to bind Grb2 and simultaneously recruit c-Cbl. However, recapitulation of either the Y1144 or Y1226/7 tyrosine phosphorylation site to the NYPD mutant restored the interaction between ErbB2 and Cbl-G306E.

To explore whether deletion of the Grb2-SH3 binding site within the C-terminal region of c-Cbl would affect the coupling of ErbB2 and c-Cbl, the Cbl-ΔPro mutant was co-expressed with ErbB2 mutants. Based on the results described above for the ErbB2-NYPD mutant, which no longer recruit Grb2, it was expected that the Cbl-ΔPro mutant would not be able to associate with ErbB2 without first recruiting Grb2 to act as an adapter. However, the results were very much intriguing and interesting. Unexpectedly, the Cbl-ΔPro mutant was still able to associate with ErbB2 (Figure 2B), suggesting the possibility of a Grb2-independent interaction. Moreover, when the ErbB2-Y1117F mutant was co-expressed with the Cbl-ΔPro mutant, the ErbB2/Cbl interaction was nearly abolished. That is, Y1117 appeared to play a role in mediating c-Cbl association with ErbB2, at least under these conditions.

Indeed, when only the N-terminal portion of c-Cbl (Cbl-N) containing the TKB region was expressed, this truncated Cbl protein was able to associate with ErbB2, independent of any of the five C-terminal tyrosine autophosphorylation sites since the ErbB2-NYPD mutant still bound to Cbl-N (Figure 2C). Again, expression of the ErbB2-Y117F mutation had deleterious effects on the binding of Cbl-N to the receptor. Conversely, mutation of the TKB site in the truncated Cbl-N variant, Cbl-N(G306E), this was unable to associate with ErbB2 or any of the mutants. The weak band present in the blot is likely due to non-specific interactions as a consequence of expressing high levels of ErbB2 and Cbl mutants. Together, these results suggest that the primary mode of interaction between ErbB2 and c-Cbl is Grb2 dependent and requires the presence of ErbB2-Y1144 or ErbB2-Y1226/7 and the Cbl proline-rich domain. A secondary direct

Figure 2. Dual binding modes for the Cbl/ErbB2 interaction.

(A) Diagrammatic representation of c-Cbl and the different mutants used in this study. ΔPro contains a deletion in the proline- rich region (PRO). G306E is a point mutation in the Tyrosine Kinase Binding (TKB) region, which includes the SH2 domain. Cbl-N is a C-terminally truncated protein containing only the TKB domain. N(G306E) is the truncated Cbl protein with the G306E point mutation. The RING finger domain is also shown. "Y" represents the tyrosine phosphorylation sites in the C-terminus of the Cbl protein. **(B)** Mutant Cbl proteins can still associate with ErbB2. The Cbl ΔPro mutant can still associate with ErbB2 except with the Y1117F mutant. When the Cbl-G306E mutant is expressed, it can interact with ErbB2 except when the tyrosine phosphorylation sites are mutated in the NYPD mutant. **(C)** The truncated Cbl protein, Cbl-N, associates with the ErbB2 mutants but not with the Y1117F mutant. Point mutation in the TKB region of the Cbl-N truncated protein, Cbl-N(G306E), resulted in the ablation of ErbB2/Cbl interaction, independent of ErbB2 tyrosine phosphorylation status. Arrowheads point to the bands that correspond to the size of the Cbl mutant protein.


A
TKB
RING
PRO
c-Cbl
ΔPro
G306E
G306E
Cbl-N
G306E
N(G306E)
B
Cbl G306E
Cbl ΔPro
NT
NYPD
YB
YD
Y1117F
NT
NYPD
YB
YD
Y1117F
IP: ErbB2
IB: Cbl
IB: Cbl
C
HA-tagged
Cbl-N
N(G306E)
NT
NYPD
YB
Y1117F
NT
NYPD
YB
Y1117F
IP: ErbB2
IB: HA
IB: HA

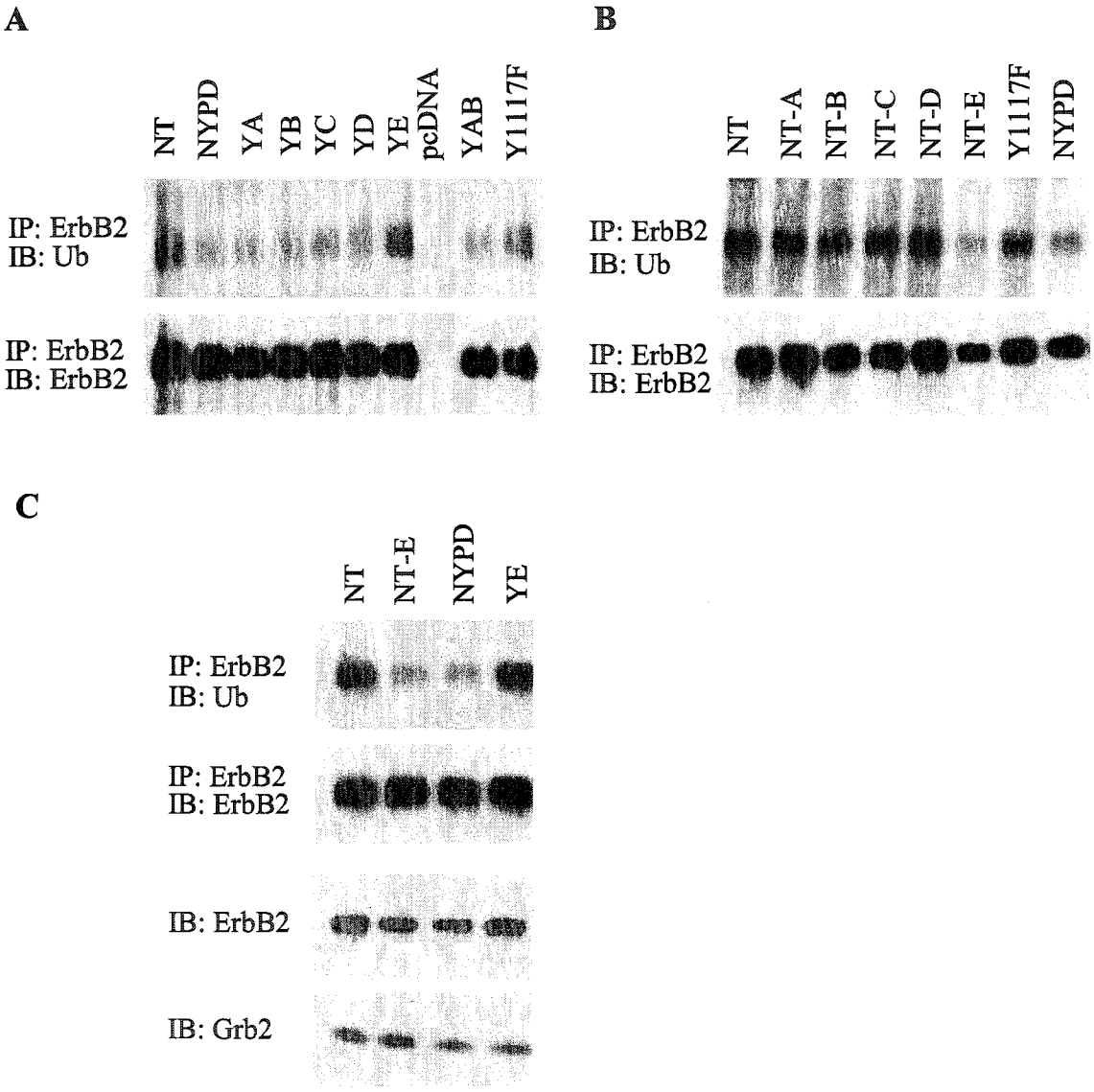
interaction between ErbB2-Y1117 and Cbl-TKB region, as suggested previously by Klapper et al. (2000), may also occur, however, we have demonstrated that this step required an alteration in the C-terminal portion of c-Cbl, either by mutation of the proline-rich domain or by complete truncation of the C-terminal portion. The TKB domain in the context of a full length wild type c-Cbl could not associate with ErbB2, perhaps requiring a conformational change to occur first.

Y1253 is required for the ubiquitylation of ErbB2

In the case of the EGFR, recruitment of c-Cbl leads to the ubiquitylation of the receptor and its subsequent downregulation. Therefore, we examined the ubiquitylation status of the different ErbB2 mutants. Surprisingly, although the Y1144 and Y1226/7 tyrosine phosphorylation sites were able to recruit c-Cbl (Figure 1B), these specific mutant ErbB2 receptors were not ubiquitylated (Figure 3A). Both the Y1144 (YB) and Y1226/7 (YD) add-back mutants were very poorly ubiquitylated when compared to the wild type activated receptor (ErbB2-NT). Conversely, point mutation of these individual sites (NT-B and NT-D) did not alter the ubiquitylation status of the receptor (Figure 2B). That is, despite the ability of the specific tyrosine autophosphorylation sites to recruit c-Cbl, this did not result in the coordinate ubiquitylation of ErbB2. Moreover, our receptor ubiquitylation data clearly points to Y1253 (YE) as the critical phosphotyrosine site that is sufficient and required for the efficient ubiquitylation of ErbB2. Restoration of Y1253 (YE add-back) to the ubiquitylation deficient NYPD mutant recapitulated the complete ubiquitylation of the ErbB2 receptor (Figure 3A and C). Conversely, single point

Figure 3. Ubiquitylation status of ErbB2 mutants

Ubiquitylation status of either (A) ErbB2 tyrosine add-back mutants or (B) ErbB2 tyrosine point mutants, as indicated. Ubiquitin blots were stripped and re-blotted to determine ErbB2 levels (lower panels). Low background level of ubiquitylation was detected consistently for all mutant ErbB2 expressed. (C) Y1253 (YE) mediated ubiquitylation was re-confirmed. The NT-E point mutant or the NYPD mutant was not efficiently ubiquitylated in the absence of Y1253. The level of ErbB2 mutants expressed in cells was detected by immunoblotting. Grb2 was used as an internal control for levels.


A
NT
NYPD
YA
YB
YC
YD
YE
pcDNA
YAB
Y1117F
IP: ErbB2
IB: Ub
IP: ErbB2
IB: ErbB2
B
NT
NT-A
NT-B
NT-C
NT-D
NT-E
Y1117F
NYPD
IP: ErbB2
IB: Ub
IP: ErbB2
IB: ErbB2
C
NT
NT-E
NYPD
YE
IP: ErbB2
IB: Ub
IP: ErbB2
IB: ErbB2
IB: ErbB2
IB: Grb2

mutation of Y1253 in the NT-E mutant resulted in a receptor that was very poorly ubiquitylated relative to ErbB2-NT (Figure 3B and C).

Despite the fact that ErbB2 was ubiquitylated, there was no indication that this modification resulted in the downregulation or turnover of the receptor. The presence of Y1253 and the ability of the mutant receptors to be ubiquitylated did not have any bearing on the level of ErbB2 detected (Figure 3C). Taken together, these results suggest that the ubiquitylation of ErbB2 mediated by tyrosine 1253 within the C-terminal tail of ErbB2 has no negative regulatory effect on the receptor.

ErbB2 is monoubiquitylated, not polyubiquitylated

Until recently, it was thought that RTK recruitment of c-Cbl lead to the polyubiquitylation of the receptor resulting in lysosomal degradation rather than proteosomal degradation. During our investigation, it was reported that the EGFR and PDGFR were monoubiquitylated and that this was sufficient to induce receptor internalization and lysosomal degradation (Haglund et al., 2003; Mosesson et al., 2003). To this end, we examined more closely the ubiquitylation status of ErbB2 to determine whether the receptor was monoubiquitylated (mono-Ub) or polyubiquitylated (poly-Ub). The distinction between the two post-translational modifications being that poly-Ub leads to protein degradation through the proteosomal complex whereas mono-Ub marks the receptor for cytoplasmic trafficking (Hicke, 2001). To distinguish between mono-Ub and poly-Ub of ErbB2, ErbB2 immunoprecipitates were immunoblotted using anti-ubiquitin (clone P4D1), which can detect both mono-Ub and poly-Ub, or anti-ubiquitin (clone

FK1), which can only detect poly-Ub proteins. Note that these are also the same anti-ubiquitin clones used by Haglund et al. (2003) to delineate the ubiquitin status of the EGFR and PDGFR. We transfected 293T cells with plasmids expressing the indicated ErbB2 mutants, prepared lysates from these cells, immunoprecipitated for ErbB2, then immunoblotted for ubiquitin. Clone FK1 did not detect any poly-Ub ErbB2 (Figure 4B) whereas clone P4D1 was able to detect ubiquitylated ErbB2 (Figure 4A). These findings suggest that ErbB2 is indeed monoubiquitylated and not polyubiquitylated. Note also that there is only a small shift in the band for monoubiquitylated ErbB2 relative to the band that appears after stripping the ubiquitin blot and immunoblotting for ErbB2 (Figure 4A, compare top and bottom panels), rather than a large up-smearing band that is typical of polyubiquitylated proteins.

Tyrosine kinase region protects ErbB2 from c-Cbl

It was puzzling as to why ErbB2 recruitment and tyrosine phosphorylation of c-Cbl, unlike for the EGFR, did not result in the concomitant ubiquitylation of the receptor. ErbB2 appeared to be protected from the downregulatory effects of c-Cbl. Since it was recently reported that c-Src could promote the destruction of c-Cbl (Bao et al., 2003) and c-Src interacts directly with ErbB2 (Muthuswamy and Muller, 1995b), we decided to examine whether this c-Src/c-Cbl interaction had any bearing on receptor ubiquitylation. To this end, a chimeric EGFR/ErbB2 receptor was previously generated where the tyrosine kinase domain of the EGFR was swapped with the tyrosine kinase region of ErbB2 to generate the EGFR-TK mutant (Figure 5A). This mutant was used to

Figure 4. ErbB2 is monoubiquitylated

Cell lysates containing oncogenic ErbB2 were immunoprecipitated to determine the nature of the ubiquitylation of the receptor. Membranes were immunoblotted for ubiquitylation using either **(A)** clone P4D1, which detects both mono- and poly-ubiquitylated proteins or **(B)** clone FK1, which detects only polyubiquitin chains. Membranes were subsequently stripped and ErbB2 levels were detected (lower panels). NT lysate was used as a control for antibody detection.

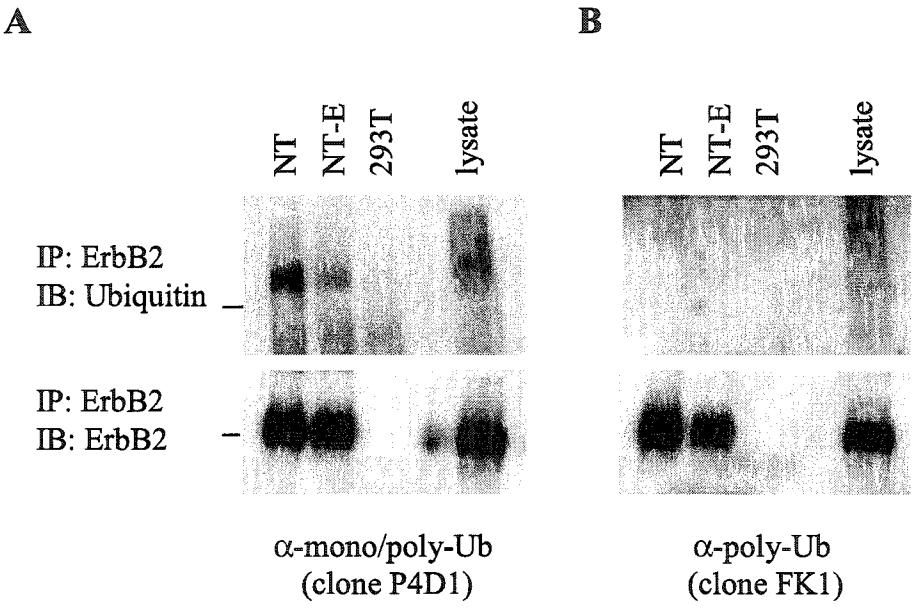
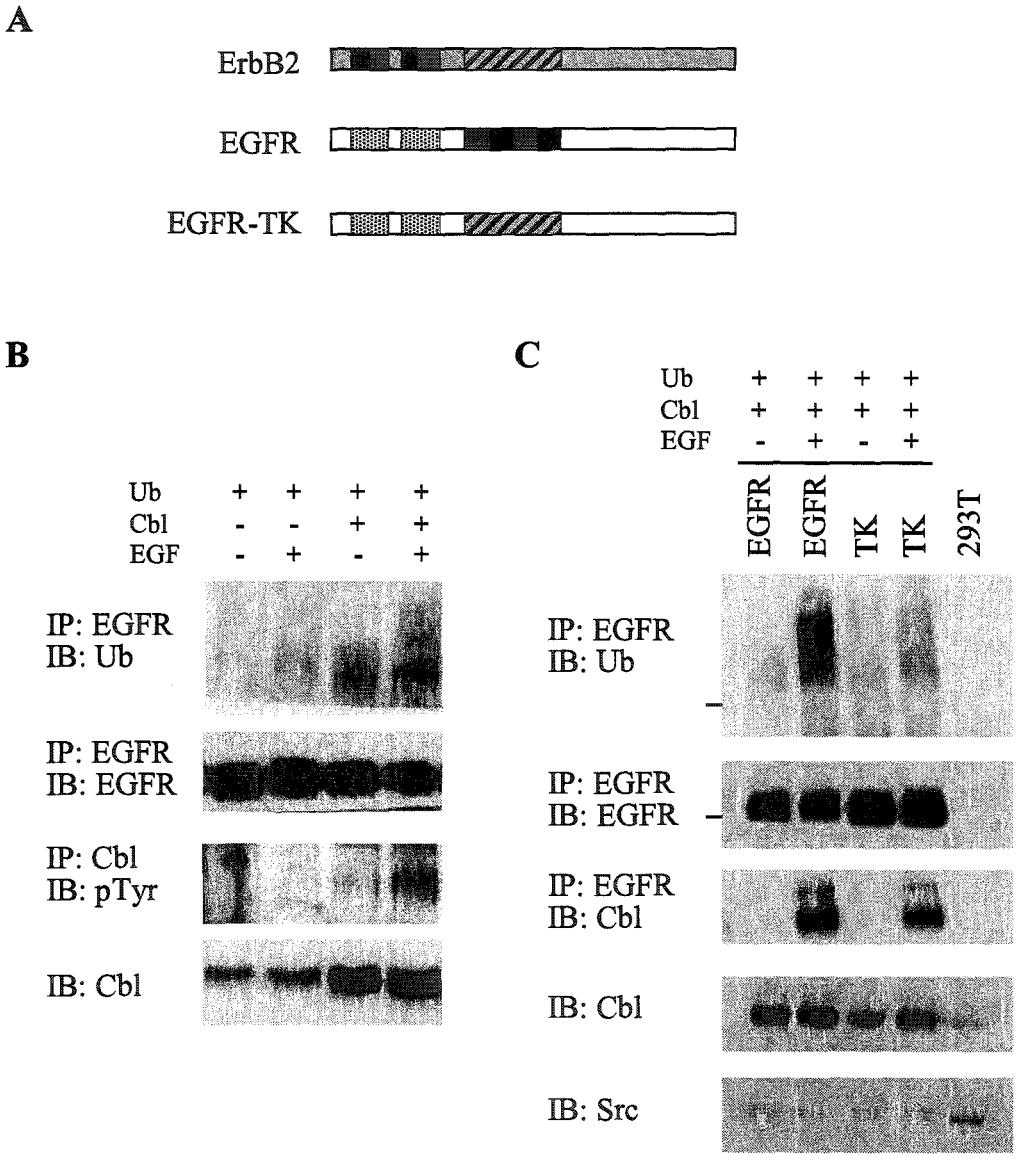

A
B
NT
NT-E
293T
lysate
IP: ErbB2
IB: Ubiquitin
IP: ErbB2
IB: ErbB2
α-mono/poly-Ub
(clone P4D1)
α-poly-Ub
(clone FK1)

Figure 5. The TK region determines sensitivity to Cbl mediated ubiquitylation.

(A) Diagrammatic representation of ErbB2, EGFR and the EGFR-TK mutant. The tyrosine kinase domain of the EGFR was swapped with the analogous region in ErbB2 to create the EGFR-TK mutant. 293T cells were co-transfected with plasmids encoding EGFR or the TK mutant and with (+) or without (-) plasmids encoding ubiquitin (Ub) and/or c-cbl (Cbl). **(B)** The ubiquitylation of the EGFR is increased upon EGF stimulation and in the presence of ectopic Cbl expression. EGF stimulation also increases the level of tyrosine phosphorylated Cbl protein. **(C)** Unlike the EGFR, the TK mutant is not efficiently ubiquitylated upon EGF stimulation, although both receptors are equally capable of recruiting Cbl.


A
ErbB2
EGFR
EGFR-TK
B
Ub + + + +
Cbl - - + +
EGF - + - +
IP: EGFR
IB: Ub
IP: EGFR
IB: EGFR
IP: Cbl
IB: pTyr
IB: Cbl
C
Ub + + + +
Cbl + + + +
EGF - + - +
EGFR
EGFR
TK
TK
293T
IP: EGFR
IB: Ub
IP: EGFR
IB: EGFR
IP: EGFR
IB: Cbl
IB: Cbl
IB: Src

demonstrate that c-Src interacts directly and specifically with the tyrosine kinase domain of ErbB2 and not with EGFR. The EGFR-TK mutant containing the ErbB2 kinase domain within the context of EGFR was able to co-immunoprecipitate with c-Src whereas the wild type EGFR could not (Kim and Muller, unpublished).

Therefore, we hypothesized that the direct interaction of c-Src with ErbB2 rendered the receptor refractory to c-Cbl mediated ubiquitylation. Figure 5B shows the c-Cbl dependent ubiquitylation of the EGFR, which was significantly elevated upon EGF stimulation and corresponded to an increased level of tyrosine phosphorylated c-Cbl. Strikingly, the TK mutant was only very weakly ubiquitylated upon EGF stimulation when compared to wild type EGFR (Figure 5C). This difference in receptor ubiquitylation occurred despite the equivalent ability of both the EGFR and TK mutant receptors to recruit c-Cbl upon EGF stimulation (Figure 5C). Therefore, the differential ability of c-Cbl to mediate the ubiquitylation of the EGFR but not ErbB2 lies in the differences in their tyrosine kinase domain. This difference lies in the ability of the ErbB2 tyrosine kinase region to directly recruit c-Src, whereas the EGFR alone is unable to interact with c-Src.

Discussion

Upon ligand binding, the EGFR recruits c-Cbl via tyrosine 1045 (Levkowitz et al., 1999), which then promotes ubiquitylation and downregulation of the receptor. This effect may be enhanced by the supplementary or surrogate Grb2-mediated recruitment of c-Cbl to the EGFR (Waterman et al., 2002). Similarly, the Met receptor can recruit c-Cbl

by two mechanisms. Met-Y1003 interacts directly with the TKB domain of c-Cbl whereas Met-Y1356, the Grb2 binding site, is required to associate with the proline-rich region of c-Cbl (Peschard et al., 2001). Other receptors, including the PDGFR, CSF-1R, and Ron, also associate with and are downregulated by the c-Cbl protein (Lee et al., 1999; Miyake et al., 1999; Penengo et al., 2003). Taken together, the ability of activated receptors to recruit the c-Cbl mediated ubiquitylation system ensures that the activity of the receptor is regulated by internalization and subsequent degradation in the lysosome. Conversely, mutant variants of these receptors that escape c-Cbl downregulation renders them oncogenic (Peschard and Park, 2003).

Although similar in structure, the ErbB2 receptor tyrosine kinase is clearly distinct from the EGFR in many ways, including its function and activity. Here we provide further examples of such differences that distinguish ErbB2 from the EGFR (and other RTKs), which may contribute to the oncogenic potency of the ErbB2 receptor. Our data suggests that the primary mode for the association of ErbB2 with c-Cbl is not direct but rather it is dependent upon the presence of tyrosine autophosphorylation sites that can associate (directly or indirectly) with the Grb2 adapter protein (ErbB2-Y1144 and Y1226/7) (Dankort et al., 1997). Thus, it is likely that the recruitment of c-Cbl by ErbB2 is dependent on Grb2 since c-Cbl and Grb2 exist constitutively in a complex together (Anderson et al., 1997; Buday et al., 1996; Donovan et al., 1996). The SH3 domain of Grb2 recognizes and is bound to the proline-rich region in the C-terminal portion of c-Cbl whereas the TKB region of c-Cbl binds directly to a phosphotyrosine protein substrate.

Consistent with the interaction of other RTKs with c-Cbl, we also observed a direct association between the N-terminal portions of c-Cbl, containing the TKB region, specifically with ErbB2-Y1117. ErbB2-Y1117 is the site in the rat ErbB2 sequence that is orthologous to the human ErbB2-Y1112, which was previously identified as the putative c-Cbl binding site (Klapper et al., 2000). This tyrosine site and its surrounding sequences align with the region surrounding EGFR-Y1045, the direct c-Cbl binding site in the EGFR (Levkowitz et al., 1999). Interestingly, it is important to note that we could not detect the binding of full length wild type c-Cbl to ErbB2-Y1117. The Y1117F mutation did not abrogate the receptor's interaction with c-Cbl, nor did mutation of the TKB domain (G306E) in the context of a full length c-Cbl protein. Instead, only the C-terminal mutant (Cbl-ΔPro) or the C-terminally truncated Cbl protein (Cbl-N) could associate with ErbB2-Y1117. In the report by Klapper et al. (2000) where they describe the initial identification of this putative direct binding site, they only demonstrated the association of v-Cbl, also a truncated protein similar to Cbl-N, to ErbB2. Taken together, these data suggests that a conformational change or the release of an auto-inhibitory mechanism is necessary for the TKB region of c-Cbl to bind to Y1117. In the model proposed by Peschard et al. (2001) for Met-Cbl interactions, they suggest that c-Cbl is first recruited to Met in a Grb2 dependent manner, followed by a phosphorylation dependent conformational change that allows the TKB region to target its direct binding site at tyrosine 1003. It is also conceivable that the direct interaction between full length c-Cbl and ErbB2-Y1117, whether or not it is secondary to that mediated by Grb2, is very weak and/or transient, thus we are unable to detect this interaction. Further evidence to

suggest that this direct binding does not occur is demonstrated by the lack of effect of the Y1117F mutant on ErbB2-mediated cellular transformation (H.Kim and W.J. Muller, unpublished data).

Despite the ability of ErbB2 to associate with c-Cbl in a Grb2 dependent manner and the potential for the TKB region of c-Cbl to bind directly to Y1117, one key difference from other RTKs is that from our studies ErbB2 is completely refractory to c-Cbl mediated receptor downregulation. Previously, another group was able to demonstrate c-Cbl mediated ubiquitylation and downregulation of ErbB2 (Levkowitz et al., 2000). The nature of this discrepancy cannot be reconciled but may be due to differences in expression levels or the use of different cell lines and the effects may be cell-type dependent. Our results clearly demonstrate that the ubiquitylation status of ErbB2 does not correlate with its ability to associate with c-Cbl. Neither Y1144 nor Y1226/7 could restore the ubiquitylation status to a ubiquitylation defective ErbB2-NYPD mutant. In fact, it is Y1253 that is responsible for the ubiquitylation of ErbB2 (discussed below). Moreover, there is no indication from previous cellular transformation assays or from MMTV-YB and MMTV-YD transgenic mice that this Grb2-dependent recruitment of c-Cbl results in any negative or inhibitory effect on ErbB2 signaling (Dankort et al., 2001b; Dankort et al., 1997). Thus, consistent with results from studies on the Ron and Met RTKs (Penengo et al., 2003; Peschard et al., 2001), the Grb2-dependent recruitment of c-Cbl to the ErbB2 receptor alone is not sufficient to lead to the downregulation of the receptor. A direct interaction with the TKB region of c-Cbl is also necessary, but since we cannot detect the association of full

length c-Cbl to the previously identified direct binding site, this anomaly may explain the unique ability of ErbB2 to escape c-Cbl mediated ubiquitylation. This is consistent with a previous report suggesting the ErbB2 is resistant to c-Cbl mediated degradation (Muthuswamy et al., 1999).

Perhaps the ErbB2 receptor has evolved to become such a potent signaling unit by losing this c-Cbl mediated regulatory effect. It also may not require such a system. We have shown that there exists an alternate negative regulatory mechanism for the ErbB2 receptor, which is dependent on tyrosine 1028 and functions in both cellular transformation and in development (Dankort et al., 1997; Chan et al., submitted 2004). As for the other ErbB family members, ErbB3 and ErbB4 also do not associate with c-Cbl but are ubiquitylated by another ubiquitin ligase, the Nrdp1/FLRF protein, and targeted for proteosomal degradation (Diamonti et al., 2002; Qiu and Goldberg, 2002).

So how does ErbB2 resist the action of c-Cbl? One possible molecular mechanism that may explain this is mediated by the c-Src protein kinase. Recently it was reported that Src promotes the destruction of c-Cbl by catalyzing the tyrosine phosphorylation of c-Cbl, which subsequently leads to self-ubiquitylation and proteosomal degradation (Bao et al., 2003; Yokouchi et al., 2001). Therefore, it may be inferred that the recruitment of c-Src proximal to a receptor may interfere with the action of c-Cbl on the receptor. Indeed, c-Src can associate directly with ErbB2 but not with the EGFR (Muthuswamy and Muller, 1995b). Moreover, we have recently demonstrated that this direct interaction occurs specifically with ErbB2-Y882 within the tyrosine kinase region of ErbB2 (H. Kim and W.J. Muller, unpublished observations). When the ErbB2

tyrosine kinase domain was substituted for the corresponding region in EGFR (EGFR-TK mutant), this was sufficient to recapitulate the direct binding of c-Src to the chimeric EGFR receptor. Strikingly, we demonstrated in this report that this substitution event renders the TK mutant refractory to c-Cbl mediated ubiquitylation and downregulation, despite its consistent ability to recruit c-Cbl. Therefore, these results are in agreement with our hypothesis that the recruitment of c-Src can protect the receptor from the action of c-Cbl and this is the underlying difference between ErbB2 and EGFR sensitivity to c-Cbl. It may also explain the synergistic potency of the ErbB2/c-Src tandem (Muthuswamy and Muller, 1995a; Muthuswamy et al., 1994).

In addition to its negative regulatory effect on RTKs, c-Cbl is a multi-adapter protein that can interact with many cellular proteins (Tsygankov et al., 2001) such that it may exert a positive signaling role downstream of ErbB2. For example, Cbl may recruit the Crk family of proteins, which leads to activation of the JNK pathway (Garcia-Guzman et al., 2000) and cytoskeletal events or it may play a role in regulating the epithelial-mesenchymal transition by promoting the breakdown of adherens junctions and allowing cell spreading and dispersion (Fournier et al., 2000). However, at this point the exact purpose for the ErbB2/c-Cbl interaction remains unresolved.

Although we did not observe c-Cbl mediated ubiquitylation of ErbB2, we were surprised to learn that the receptor was still ubiquitylated. The molecular detail mediating this effect has not been determined at this point however we did identify that tyrosine Y1253 is entirely responsible for mediating the robust ubiquitylation of ErbB2. In the absence of Y1253, as with several of the ErbB2 tyrosine phosphorylation mutants

studied, the receptor was not ubiquitylated or very poorly ubiquitylated above background level. Note that the basal level ubiquitylation that is observed consistently with all of the ErbB2 mutants likely is due to the high expression level of ErbB2 and may be non-specific. It may also encompass endogenous ErbB2 that has been transactivated by ectopic ErbB2 oncogenic mutants.

The exact role of the ubiquitylation of ErbB2 is not clear, except that it does not appear to function in its typical role to target the protein for lysosomal or proteosomal degradation. We did not detect by standard immunoblotting methods any adverse effects of this ubiquitin tag on ErbB2 protein levels. Interestingly, our data clearly discriminates ErbB2 ubiquitylation as being monoubiquitylated rather than polyubiquitylated. The EGFR also was recently determined to be monoubiquitylated and not polyubiquitylated as previously thought. This monoubiquitylation of EGFR is sufficient to account for the c-Cbl mediated receptor internalization, endosomal delivery and lysosomal degradation of the receptor (Haglund et al., 2003; Mosesson et al., 2003). This is a very important distinction since, in general, polyubiquitylation targets a protein for degradation via the proteosome complex (Deveraux et al., 1994), which is not the pathway undertaken by ubiquitylated RTKs, whereas monoubiquitylation may lead to alternative pathways regulating the location and activity of proteins including internalization and endosomal sorting, lysosomal targeting, and possibly other yet unidentified intracellular trafficking signals (Hicke, 2001).

Thus, consistent with our observation that Y1253-mediated monoubiquitylation does not necessarily lead to receptor downregulation, cellular transformation assays

involving Y1253 suggest that there is no negative effects of Y1253 on ErbB2 activity (Dankort et al., 1997). Moreover, MMTV-YE transgenic animals develop very rapid and aggressive mammary tumors that are distinct from other MMTV-ErbB2 driven tumors (L. Jung and W.J. Muller, unpublished observations), again suggesting that the monoubiquitylated receptor is not targeted for lysosomal degradation. It is possible that the monoubiquitin signal still promotes ErbB2 internalization and endocytosis since a previous study suggested that Y1253 alone was sufficient for ErbB2 endocytosis (Gilboa et al., 1995), but it is somehow shunted away from the lysosomal compartment. We propose that the monoubiquitylation of ErbB2 targets the receptor to an alternate subcellular location. This would bring it proximal to a different set of cellular proteins that distinguishes its activity from ErbB2 signaling from the plasma membrane. Interestingly, ErbB2-Y1253 is predominantly tyrosyl-phosphorylated in a large percentage of human breast cancer samples overexpressing ErbB2 (Eppenberger-Castori et al., 2001). These results suggest a unique and essential role for Y1253 in ErbB2-mediated signaling. Thus, it would be essential and useful to understand the unique signaling and the molecular mechanisms of action surrounding ErbB2-Y1253 since it may have important clinical value.

Taken together, the evidence clearly points to a unique role and function of ErbB2 that distinguishes it from other receptor tyrosine kinases and possibly contributing to its oncogenecity. Molecular and genetic dissection of ErbB2 signaling to understand precisely the signaling properties of ErbB2 and its individual signaling components sites will require continued experimentation.

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References

Akiyama, T., Matsuda, S., Namba, Y., Saito, T., Toyoshima, K. and Yamamoto, T. (1991). The transforming potential of the c-erbB-2 protein is regulated by its autophosphorylation at the carboxyl-terminal domain. *Mol Cell Biol* **11**, 833-42.

Anderson, S. M., Burton, E. A. and Koch, B. L. (1997). Phosphorylation of Cbl following stimulation with interleukin-3 and its association with Grb2, Fyn, and phosphatidylinositol 3-kinase. *J Biol Chem* **272**, 739-45.

Bao, J., Gur, G. and Yarden, Y. (2003). Src promotes destruction of c-Cbl: implications for oncogenic synergy between Src and growth factor receptors. *Proc Natl Acad Sci U S A* **100**, 2438-43.

Bargmann, C. I., Hung, M. C. and Weinberg, R. A. (1986). Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell* **45**, 649-57.

Buday, L., Khwaja, A., Sipeki, S., Farago, A. and Downward, J. (1996). Interactions of Cbl with two adapter proteins, Grb2 and Crk, upon T cell activation. *J Biol Chem* **271**, 6159-63.

Burden, S. and Yarden, Y. (1997). Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron* **18**, 847-55.

Dankort, D., Maslikowski, B., Warner, N., Kanno, N., Kim, H., Wang, Z., Moran, M. F., Oshima, R. G., Cardiff, R. D. and Muller, W. J. (2001). Grb2 and Shc adapter proteins play distinct roles in Neu (ErbB-2)-induced mammary tumorigenesis: implications for human breast cancer. *Mol Cell Biol* **21**, 1540-51.

Dankort, D. L., Wang, Z., Blackmore, V., Moran, M. F. and Muller, W. J. (1997). Distinct tyrosine autophosphorylation sites negatively and positively modulate neu-mediated transformation. *Mol Cell Biol* **17**, 5410-25.

Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994). A 26 S protease subunit that binds ubiquitin conjugates. *J Biol Chem* **269**, 7059-61.

Diamonti, A. J., Guy, P. M., Ivanof, C., Wong, K., Sweeney, C. and Carraway, K. L., 3rd. (2002). An RBCC protein implicated in maintenance of steady-state neuregulin receptor levels. *Proc Natl Acad Sci U S A* **99**, 2866-71.

Donovan, J. A., Ota, Y., Langdon, W. Y. and Samelson, L. E. (1996). Regulation of the association of p120cbl with Grb2 in Jurkat T cells. *J Biol Chem* **271**, 26369-74.

Eppenberger-Castori, S., Kueng, W., Benz, C., Caduff, R., Varga, Z., Bannwart, F., Fink, D., Dieterich, H., Hohl, M., Muller, H. et al. (2001). Prognostic and predictive significance of ErbB-2 breast tumor levels measured by enzyme immunoassay. *J Clin Oncol* **19**, 645-56.

Fazioli, F., Kim, U. H., Rhee, S. G., Molloy, C. J., Segatto, O. and Di Fiore, P. P. (1991). The erbB-2 mitogenic signaling pathway: tyrosine phosphorylation of phospholipase C-gamma and GTPase-activating protein does not correlate with erbB-2 mitogenic potency. *Mol Cell Biol* **11**, 2040-8.

Fournier, T. M., Lamorte, L., Maroun, C. R., Lupher, M., Band, H., Langdon, W. and Park, M. (2000). Cbl-transforming variants trigger a cascade of molecular alterations that lead to epithelial mesenchymal conversion. *Mol Biol Cell* **11**, 3397-410.

Fukazawa, T., Miyake, S., Band, V. and Band, H. (1996). Tyrosine phosphorylation of Cbl upon epidermal growth factor (EGF) stimulation and its association with EGF receptor and downstream signaling proteins. *J Biol Chem* **271**, 14554-9.

Garcia-Guzman, M., Larsen, E. and Vuori, K. (2000). The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: a role for the adaptor protein Crk. *Oncogene* **19**, 4058-65.

Gilboa, L., Ben-Levy, R., Yarden, Y. and Henis, Y. I. (1995). Roles for a cytoplasmic tyrosine and tyrosine kinase activity in the interactions of Neu receptors with coated pits. *J Biol Chem* **270**, 7061-7.

Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P. and Dikic, I. (2003). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat Cell Biol* **5**, 461-6.

Hazan, R., Margolis, B., Dombalagian, M., Ullrich, A., Zilberstein, A. and Schlessinger, J. (1990). Identification of autophosphorylation sites of HER2/neu. *Cell Growth Differ* **1**, 3-7.

Hicke, L. (2001). Protein regulation by monoubiquitin. *Nat Rev Mol Cell Biol* **2**, 195-201.

Hynes, N. E. and Stern, D. F. (1994). The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta* **1198**, 165-84.

Jallal, B., Schlessinger, J. and Ullrich, A. (1992). Tyrosine phosphatase inhibition permits analysis of signal transduction complexes in p185HER2/neu-overexpressing human tumor cells. *J Biol Chem* **267**, 4357-63.

Janes, P. W., Daly, R. J., deFazio, A. and Sutherland, R. L. (1994). Activation of the Ras signalling pathway in human breast cancer cells overexpressing erbB-2. *Oncogene* **9**, 3601-8.

Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T. and Liu, Y. C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **286**, 309-12.

Kavanaugh, W. M., Turck, C. W. and Williams, L. T. (1995). PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science* **268**, 1177-9.

Klapper, L. N., Waterman, H., Sela, M. and Yarden, Y. (2000). Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. *Cancer Res* **60**, 3384-8.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C. and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-8.

Lee, P. S., Wang, Y., Dominguez, M. G., Yeung, Y. G., Murphy, M. A., Bowtell, D. D. and Stanley, E. R. (1999). The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. *Embo J* **18**, 3616-28.

Levkowitz, G., Oved, S., Klapper, L. N., Harari, D., Lavi, S., Sela, M. and Yarden, Y. (2000). c-Cbl is a suppressor of the neu oncogene. *J Biol Chem* **275**, 35532-9.

Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A. et al. (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol Cell* **4**, 1029-40.

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B. and Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* **12**, 3663-74.

Lill, N. L., Douillard, P., Awwad, R. A., Ota, S., Lupher, M. L., Jr., Miyake, S., Meissner-Lula, N., Hsu, V. W. and Band, H. (2000). The evolutionarily conserved N-terminal region of Cbl is sufficient to enhance down-regulation of the epidermal growth factor receptor. *J Biol Chem* **275**, 367-77.

Luttrell, D. K., Lee, A., Lansing, T. J., Crosby, R. M., Jung, K. D., Willard, D., Luther, M., Rodriguez, M., Berman, J. and Gilmer, T. M. (1994). Involvement of

pp60c-src with two major signaling pathways in human breast cancer. *Proc Natl Acad Sci U S A* **91**, 83-7.

Meisner, H. and Czech, M. P. (1995). Coupling of the proto-oncogene product c-Cbl to the epidermal growth factor receptor. *J Biol Chem* **270**, 25332-5.

Meng, W., Sawasdikosol, S., Burakoff, S. J. and Eck, M. J. (1999). Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase. *Nature* **398**, 84-90.

Miyake, S., Mullane-Robinson, K. P., Lill, N. L., Douillard, P. and Band, H. (1999). Cbl-mediated negative regulation of platelet-derived growth factor receptor-dependent cell proliferation. A critical role for Cbl tyrosine kinase-binding domain. *J Biol Chem* **274**, 16619-28.

Mosesson, Y., Shtiegman, K., Katz, M., Zwang, Y., Vereb, G., Szollosi, J. and Yarden, Y. (2003). Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. *J Biol Chem* **278**, 21323-6.

Muthuswamy, S. K., Gilman, M. and Brugge, J. S. (1999). Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. *Mol Cell Biol* **19**, 6845-57.

Muthuswamy, S. K. and Muller, W. J. (1995a). Activation of Src family kinases in Neu-induced mammary tumors correlates with their association with distinct sets of tyrosine phosphorylated proteins in vivo. *Oncogene* **11**, 1801-10.

Muthuswamy, S. K. and Muller, W. J. (1995b). Direct and specific interaction of c-Src with Neu is involved in signaling by the epidermal growth factor receptor. *Oncogene* **11**, 271-9.

Muthuswamy, S. K., Siegel, P. M., Dankort, D. L., Webster, M. A. and Muller, W. J. (1994). Mammary tumors expressing the neu proto-oncogene possess elevated c-Src tyrosine kinase activity. *Mol Cell Biol* **14**, 735-43.

Nakatsu, F., Sakuma, M., Matsuo, Y., Arase, H., Yamasaki, S., Nakamura, N., Saito, T. and Ohno, H. (2000). A Di-leucine signal in the ubiquitin moiety. Possible involvement in ubiquitination-mediated endocytosis. *J Biol Chem* **275**, 26213-9.

Pawson, T. (1995). Protein modules and signalling networks. *Nature* **373**, 573-80.

Peles, E., Lamprecht, R., Ben-Levy, R., Tzahar, E. and Yarden, Y. (1992). Regulated coupling of the Neu receptor to phosphatidylinositol 3'-kinase and its release by oncogenic activation. *J Biol Chem* **267**, 12266-74.

Peles, E., Levy, R. B., Or, E., Ullrich, A. and Yarden, Y. (1991). Oncogenic forms of the neu/HER2 tyrosine kinase are permanently coupled to phospholipase C gamma. *Embo J* **10**, 2077-86.

Penengo, L., Rubin, C., Yarden, Y. and Gaudino, G. (2003). c-Cbl is a critical modulator of the Ron tyrosine kinase receptor. *Oncogene* **22**, 3669-79.

Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y. and Park, M. (2001). Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol Cell* **8**, 995-1004.

Peschard, P. and Park, M. (2003). Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases. *Cancer Cell* **3**, 519-23.

Qiu, X. B. and Goldberg, A. L. (2002). Nrdp1/FLRF is a ubiquitin ligase promoting ubiquitination and degradation of the epidermal growth factor receptor family member, ErbB3. *Proc Natl Acad Sci U S A* **99**, 14843-8.

Scott, G. K., Dodson, J. M., Montgomery, P. A., Johnson, R. M., Sarup, J. C., Wong, W. L., Ullrich, A., Shepard, H. M. and Benz, C. C. (1991). p185HER2 signal transduction in breast cancer cells. *J Biol Chem* **266**, 14300-5.

Segatto, O., Pelicci, G., Giuli, S., Digiesi, G., Di Fiore, P. P., McGlade, J., Pawson, T. and Pelicci, P. G. (1993). Shc products are substrates of erbB-2 kinase. *Oncogene* **8**, 2105-12.

Shih, S. C., Sloper-Mould, K. E. and Hicke, L. (2000). Monoubiquitin carries a novel internalization signal that is appended to activated receptors. *Embo J* **19**, 187-98.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177-82.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**, 707-12.

Stein, D., Wu, J., Fuqua, S. A., Roonprapunt, C., Yajnik, V., D'Eustachio, P., Moskow, J. J., Buchberg, A. M., Osborne, C. K. and Margolis, B. (1994). The SH2 domain protein GRB-7 is co-amplified, overexpressed and in a tight complex with HER2 in breast cancer. *Embo J* **13**, 1331-40.

Thien, C. B. and Langdon, W. Y. (2001). Cbl: many adaptations to regulate protein tyrosine kinases. *Nat Rev Mol Cell Biol* **2**, 294-307.

Thien, C. B., Walker, F. and Langdon, W. Y. (2001). RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation. *Mol Cell* **7**, 355-65.

Tsygankov, A. Y., Teckchandani, A. M., Feshchenko, E. A. and Swaminathan, G. (2001). Beyond the RING: CBL proteins as multivalent adapters. *Oncogene* **20**, 6382-402.

Waterman, H., Katz, M., Rubin, C., Shtiegman, K., Lavi, S., Elson, A., Jovin, T. and Yarden, Y. (2002). A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling. *Embo J* **21**, 303-13.

Waterman, H., Levkowitz, G., Alroy, I. and Yarden, Y. (1999). The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. *J Biol Chem* **274**, 22151-4.

Weiner, D. B., Liu, J., Cohen, J. A., Williams, W. V. and Greene, M. I. (1989). A point mutation in the neu oncogene mimics ligand induction of receptor aggregation. *Nature* **339**, 230-1.

Weissman, A. M. (2001). Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* **2**, 169-78.

Yokouchi, M., Kondo, T., Sanjay, A., Houghton, A., Yoshimura, A., Komiya, S., Zhang, H. and Baron, R. (2001). Src-catalyzed phosphorylation of c-Cbl leads to the interdependent ubiquitination of both proteins. *J Biol Chem* **276**, 35185-93.

Zheng, N., Wang, P., Jeffrey, P. D. and Pavletich, N. P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* **102**, 533-9.

Chapter 6

Conclusions and Future Directions

The EGFR/ErbB family of receptor tyrosine kinases is an important group of signaling molecules that play essential roles in a number of biological processes in both normal development and in disease onset and progression. Of particular interest is the ErbB2 receptor since it has been proposed to be the preferential heterodimerization partner and the master coordinator of this signaling network (Graus-Porta et al., 1997). Indeed, loss of ErbB2 expression by targeted gene disruption led to animals that died at midgestation with cardiac and nervous system defects (Lee et al., 1995), whereas overexpression of ErbB2 in the murine mammary gland, for example, resulted in multiple tumor formation with a propensity to metastasize (Guy et al., 1992). Given the evidence suggesting the importance of maintaining or regulating ErbB2, several in vitro studies were undertaken to dissect ErbB2 signaling mechanisms and function. Much of the work has focused on the catalytic domain (Qian et al., 1994a; Qian et al., 1994b; Weiner et al., 1989a) or the carboxy-terminal regulatory region of the receptor where several tyrosine autophosphorylation sites are located (Akiyama et al., 1991; Ben-Levy et al., 1994; Dankort et al., 2001a; Dankort et al., 1997; Di Fiore et al., 1990; Mikami et al., 1992; Segatto et al., 1992; Settle et al., 2003). Although the results of these studies have been extremely informative, they do carry the limitations inherent to all in vitro studies. To this end, this thesis described the development and use of a targeted knock-in strategy to

express wild type or mutant ErbB2 receptors to examine ErbB2 receptor signaling within the complexity of a physiological in vivo context.

In Chapter 3, I concluded that the ErbB2 kinase activity is absolutely essential since expression of a kinase deficient ErbB2 receptor resulted in embryonic lethality similar to *erbB2* null animals (Chan et al., 2002). That is, signaling from the ErbB2 carboxy-terminus alone is not sufficient but also requires its catalytic activity, presumably to transphosphorylate its heterodimer partners. It also suggests that the other receptors cannot compensate for the loss of ErbB2 enzymatic activity and reinforces the notion regarding ErbB2 being the central mediator of the majority of ErbB signaling. Indeed, in hemizygous animals expressing a mere 5% of wild type ErbB2 levels (Chapter 4), this was sufficient to at least rescue the cardiac trabecular defects observed in midgestation embryos associated with expression of the kinase-dead allele or the complete knock-out allele. However, this level of expression was not sufficient for the proper development of the nervous system and these animals died perinatally due to poor innervation of their diaphragm muscle. Intriguingly, when the expression level was up to 10% of wild type levels, in the case of homozygous knock-in animals, this was adequate to rescue the *erbB2*-deficient defects and the animals continued to thrive and were phenotypically normal. Taken together, a minimum threshold level of ErbB2 required for development was established at approximately 10% of wild type levels. To my knowledge, this type of information has not been established for any other RTK. An allelic series with hypomorphic level of expression or subtle mutations have been established for N-myc (Nagy et al., 1998) and FGFR1 (Partanen et al., 1998) however

these hypomorphs suffered phenotypic consequences. This begs the question – is there any significance to the broad difference in the minimum threshold level and wild type level of ErbB2 expression with respect to normal growth and maintenance?

The results identified from the analyses of the kinase-dead ErbB2 receptor and the hypomorphic *erbB2* knock-in allele is significant with respect to cancer therapy targeting the ErbB2 receptor. In particular, small molecule tyrosine kinase inhibitors targeting the ATP binding site in the kinase region of the EGFR or ErbB2 receptors have been designed and tested for use in cancer therapy with moderate success (Noonberg and Benz, 2000). From a molecular and biological perspective, due to the potency of the ErbB2 kinase domain and its demonstrated critical role in normal development (Chan et al., 2002), on the one hand targeting ErbB2 kinase activity may have dramatic positive therapeutic effects, but on the other hand complete inhibition of ErbB2 activity may carry severe side effects. Keep in mind that at least in the murine model, only 10% level of ErbB2 was sufficient for normal function so what this provides perhaps is a relatively broad window in which anti-ErbB2 therapeutics may function to adequately impede the disease-state but without critically disrupting any normal requirements for ErbB2 signaling. Whether or not targeting the kinase domain or a more specific aspect of ErbB2 signaling is better from a therapeutic stand point remains to be determined. Previous in vitro analyses and the work presented in this thesis regarding the individual tyrosine autophosphorylation sites should provide valuable information for the future design and implementation of cancer therapies with increased efficacy.

One of the drawbacks of the in vitro transformation assays in studying the role of specific signaling pathways is that the results are based on a limited one-dimensional readout - cell transformation. Therefore, the conclusion that the ErbB2 tyrosine phosphorylation sites emit overlapping and redundant signals and neither phosphorylation site is essential for transformation (Dankort et al., 1997) may not necessarily apply in a complex, multi-dimensional in vivo system. Indeed, transgenic animals expressing the MMTV-YB (Y1144 – Grb2 binding site) and MMTV-YD (Y1226/7 – Shc binding site) ErbB2 add-back mutants developed mammary tumors with different properties. YB-induced mammary tumors developed after a longer latency but metastasized at a much greater frequency (Dankort et al., 2001b). In this regard, initial observations of the Y1144F or Y1226/7F *erbB2* knock-in animals did not reveal any obvious phenotype since the animals were healthy and fertile. However, upon closer examination a subtle defect was observed in the developing cutaneous sensory nerves of the Y1226/7F knock-in animals but not in the Y1144F or other knock-in animals (Chapter 4). In the absence of Shc signaling originating from ErbB2, the cutaneous sensory nerves in E12.5 embryos developed but were disorganized and defasciculated. The consequences of this defect were not immediately apparent but it is hypothesized that these animals would experience impaired response to external sensory stimuli such as in a heat test. It would be worthy to examine Shc mutant animals (Lai and Pawson, 2000) to determine whether they experience a similar phenotype, in which case it may be concluded that Shc plays a critical role in mediating the proper development of the cutaneous sensory nerves. There is evidence that Shc signals neuronal survival and

axonal growth and one of the mediators of this action downstream of Shc is the PI3'-kinase (Atwal et al., 2000). What is striking about this is that PI3'-kinase is the major signaling pathway downstream of ErbB3 and *erbB3* deficient animals display the same sensory nerve phenotype. Nonetheless, the results presented in Chapter 4 provide additional compelling evidence reiterating the contention that Grb2 and Shc utilize distinct effector pathways. Unique signals from the other two tyrosine phosphorylation sites and their individual roles likely would be similarly revealed through the generation and examination of Y1201F and Y1253F ErbB2 knock-in animals.

Previous in vitro studies identified ErbB2-Y1028 as a negative regulatory site capable of suppressing oncogenic ErbB2 mediated transformation of fibroblasts (Dankort et al., 1997). Chapter 4 described the validation of Y1028 as a bona fide negative regulator of ErbB2 signaling in a physiological context. It is suggested from the data that Y1028 functions during mammalian development to modulate the level of ErbB2 since genetic mutation of Y1028 was sufficient to rescue the defects observed in the control, albeit hypomorphic, knock-in animals. Indeed, expression of a mutant ErbB2-Y1028F receptor in knock-in animals resulted in a 3-5 fold increase in the level of ErbB2 protein when compared to the control knock-in embryos. It would be interesting to see if the same fold increase in ErbB2 levels also would be observed if the knock-in strategy expressed wild type levels of ErbB2 instead of the threshold level in the current knock-in animals. In fact, it is predicted that overexpression of ErbB2 protein above wild type levels would result in deleterious effects on the embryos. That is, the developing embryo may be sensitive to elevated ErbB2 signaling activity, in which case, Y1028 would play a

critical role in suppressing ErbB2 levels that is favorable for viability. Consistent with this notion is that germline expression of a constitutively activated ErbB2 allele resulted in early embryonic lethality (Andrechek et al., 2004). Also, deregulation of RTKs following the loss of the binding site for c-Cbl and its downregulatory effect has been linked to a number of malignancies (Peschard and Park, 2003). In this regard, aberrant overexpression of ErbB2 has been implicated in human breast cancer and this correlates inversely with overall patient survival. So far, there have not been any reports regarding the deregulation of ErbB2 signaling caused by mutation of tyrosine 1028, per se, to implicate Y1028 mutations in human cancers. It is possible that molecules downstream of Y1028 that normally mediate ErbB2 downregulation may harbor mutations that compromise the ability to modulate ErbB2 protein levels.

As described in Chapter 4, I determined the mode of action of tyrosine 1028 on the receptor by labeling the protein then following its degradation over time. Indeed, the presence of tyrosine 1028 decreased the stability of ErbB2 and promoted the downregulation or turnover of the receptor. Conversely, mutation of Y1028 resulted in a more stable receptor with a turnover rate that was significantly less than that of a wild type receptor. How exactly Y1028 mediates the increased turnover of the receptor remains to be determined. What was intriguing to us is that Y1028 mediated downregulation of ErbB2 is independent of c-Cbl interaction and ubiquitylation of the receptor (Chapter 4 and 5). In fact, ErbB2 appeared to be completely refractory to the action of c-Cbl (Chapter 5). In contrast, it is well documented that c-Cbl binds to and mediates the ubiquitylation of the EGFR (as well as other RTKs) leading to receptor

internalization and lysosomal degradation (Thien and Langdon, 2001). Therefore, based on my results examining the ErbB2 tyrosine 1028 phosphorylation site, there exists an unidentified mechanism to attenuate ErbB2 signaling upon activation of the receptor that functions independently of the c-Cbl protein and receptor ubiquitylation.

Although the monoubiquitylation of EGFR is required for its internalization and endocytic trafficking to the lysosome (Haglund et al., 2003) and this appears to be a common theme for the majority of proteins destined for lysosomal degradation, there exists a non-ubiquitin mediated mechanism to shuttle proteins to the lysosome. The Sna3p protein in yeast (Reggiori and Pelham, 2001) and the mammalian G-protein coupled Delta Opioid Receptor (DOR) (Hislop et al., 2004; Tanowitz and Von Zastrow, 2002) are such examples of proteins that are shuttled to the lysosome without first being tagged with ubiquitin. In the case of DOR, a recently identified protein, GASP (G protein-coupled-receptor-associated sorting protein) was shown to be required to mediate this process since disruption of the DOR-GASP interaction inhibited receptor trafficking to lysosomes and promoted recycling (Whistler et al., 2002). Interestingly, EGFR receptors are also sorted and directed to the lysosome through its association with either one of two related proteins, Sorting Nexin-1 (SNX-1) or Sorting Nexin-2 (SNX-2) (Gullapalli et al., 2004; Kurten et al., 1996). The precise mechanisms and how exactly these proteins assist in intracellular sorting to the lysosome without the ubiquitin signal will require further investigation. Whether or not these mechanisms also apply to the ErbB2 receptor is unknown at this time, however, they do set the precedence for ubiquitin-independent lysosomal degradation. It is very likely that in the near future,

more and more proteins will be discovered to follow this unconventional route for receptor degradation. As for the ErbB2 receptor and its Y1028 mediated degradation, is there an SH2- or PTB-containing protein that recognizes the Y1028 phosphorylation site to mediate this effect? Perhaps tyrosine phosphorylation of Y1028 elicits a conformational change in the receptor that promotes downregulation? Does the Y1028 signal funnel the receptor to a lysosomal or proteosomal degradation pathway? Or does Y1028 signaling shunt the receptor away from the recycling endosome pathway?

In Chapter 4, I discussed how tyrosine 992 within the EGFR cytoplasmic tail shares sequence similarity and to some degree functional identity with tyrosine 1028 of ErbB2. Although candidate proteins have been identified to bind to EGFR-Y992, none of these appear to associate with ErbB2-Y1028 and/or provide an answer to the mechanism of Y1028 mediated downregulation. To this end, I have already generated a series of constructs expressing ErbB2 mutants containing a TAP tag sequence (Rigaut et al., 1999) fused in-frame with the receptor at the C-terminus for use in future/current research beyond the scope of this thesis. This TAP (Tandem Affinity Purification) method allows for the effective and rapid purification of native protein complexes expressed at physiological levels. The tag consists of two consecutive affinity units, protein A and calmodulin-binding peptide, separated by a TEV protease cleavage site. Following successive isolation and purification of the receptor-complex, participants that associate with ErbB2 may be identified by employing proteomic technologies including mass spectrometry (Link et al., 1999). By comparing profiles of the complexes formed around ErbB2 receptors with and without the tyrosine-to-phenylalanine mutation at

tyrosine-1028, that is, ErbB2-TAP versus ErbB2-Y1028F-TAP and NYPD-TAP versus YA-TAP, it is expected that this will lead to the identification of individual proteins that specifically recognize the phosphorylated Y1028 site and may play a role in Y1028 mediated attenuation of ErbB2.

A similar TAP tag approach is also planned in future work to identify candidate proteins associated with phosphorylated tyrosine 1253. The reason for this stems from a very intriguing and unexpected observation that was made regarding ErbB2-Y1253 while investigating the c-Cbl/ErbB2 association (Chapter 5). ErbB2-Y1253 was found to be solely responsible for mediating the monoubiquitylation of ErbB2 in a c-Cbl independent manner. Thus, it is desirable to identify the protein that is binding to Y1253 and recruiting the ubiquitylation machinery. However, unlike the EGFR there is no indication that a monoubiquitylated ErbB2 is attenuated by being targeted for lysosomal degradation. The YE (Y1253) add-back mutant, which is specifically ubiquitylated relative to the other add-backs, is fully capable of mediating cellular transformation to a level equal to the other transforming mutants (Dankort et al., 1997). If Y1253-mediated ubiquitylation of ErbB2 does not lead to receptor downregulation, then what role does the ubiquitin tag play on ErbB2 signaling? Further specific analyses including pulse-chase turnover assays and detailed cellular localization studies to determine the route taken by a monoubiquitylated ErbB2-Y1253 receptor will be investigated. It also would be useful to map the lysine residue(s) that become covalently linked to the ubiquitin unit and mutate these sites to determine whether a ubiquitin incompetent receptor behaves similarly as a non-ubiquitylated receptor (i.e. a Y1253F receptor).

The finding that Y1253 mediated the monoubiquitylation of ErbB2 without any effect on the stability of the receptor suggests that this may be an alternate cytoplasmic or endosomal trafficking signal. Interestingly, preliminary immunofluorescent labeling of ErbB2 in Rat-1 cell lines stably expressing the YE add-back receptor and cellular fractionation analyses has identified a significant proportion of YE add-back receptor in a subcellular location other than at the expected plasma membrane (B. Maslikowski, L. Jung, D. Zuo and W.J. Muller, unpublished observations). Strikingly, the majority of YE receptors were localized in the cytoplasm. In contrast, NYPD receptors were, as expected, localized around the plasma membrane. These results are consistent with the notion that Y1253 targets the receptor for monoubiquitylation and that this modification is required for the cytoplasmic trafficking of the receptor to a specific site. The precise mechanism and rationale for this unique trafficking of the ErbB2 receptor is currently under investigation and its results will be very exciting and informative.

In summary, I have described in this thesis the identification of several important and unique insights into various aspects of ErbB2 signaling. The kinase activity of ErbB2 is indispensable for development and cannot be compensated for by other ErbB family members. Despite this critical requirement for ErbB2, a minimum threshold level of ErbB2 expression was established at approximately 10% of wild type levels and this is sufficient for development. The regulation and maintenance of ErbB2 levels does not appear to be dependent on the association of c-Cbl with ErbB2 since this did not correlate with changes in ErbB2 levels in both in vitro and in vivo analyses. Either this is a structurally non-functional interaction between c-Cbl and ErbB2 or the receptor is

naturally protected from the action of c-Cbl since their association does not lead to the downregulation of ErbB2. Instead, to modulate ErbB2 levels, tyrosine-1028 acts through a c-Cbl and ubiquitin independent mechanism to destabilize the receptor and promote its degradation. Through the investigation of c-Cbl/ErbB2 interaction, it also led to the discovery that ErbB2 is monoubiquitylated through an unknown mechanism that is dependent on the presence of phosphotyrosine Y1253 and this may play a role in the subcellular trafficking of the receptor. Analyses of the Y1144 and Y1226/7 tyrosine phosphorylation site in vivo provided clear evidence that each of the tyrosine autophosphorylation sites in the C-terminal tail of ErbB2 signal independently and have distinct roles. These results provide precedence to generate similar *erbB2* knock-in animals to examine in vivo the functional roles of the Y1201 and Y1253 phosphorylation sites.

References

Akiyama, T., Matsuda, S., Namba, Y., Saito, T., Toyoshima, K. and Yamamoto, T. (1991). The transforming potential of the c-erbB-2 protein is regulated by its autophosphorylation at the carboxyl-terminal domain. *Mol Cell Biol* **11**, 833-42.

Alimandi, M., Wang, L. M., Bottaro, D., Lee, C. C., Kuo, A., Frankel, M., Fedi, P., Tang, C., Lippman, M. and Pierce, J. H. (1997). Epidermal growth factor and betacellulin mediate signal transduction through co-expressed ErbB2 and ErbB3 receptors. *Embo J* **16**, 5608-17.

Alroy, I. and Yarden, Y. (1997). The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett* **410**, 83-6.

Anderson, S. M., Burton, E. A. and Koch, B. L. (1997). Phosphorylation of Cbl following stimulation with interleukin-3 and its association with Grb2, Fyn, and phosphatidylinositol 3-kinase. *J Biol Chem* **272**, 739-45.

Andrechek, E. R., Hardy, W. R., Girgis-Gabardo, A. A., Perry, R. L., Butler, R., Graham, F. L., Kahn, R. C., Rudnicki, M. A. and Muller, W. J. (2002). ErbB2 is required for muscle spindle and myoblast cell survival. *Mol Cell Biol* **22**, 4714-22.

Andrechek, E. R., Hardy, W. R., Laing, M. A. and Muller, W. J. (2004). Germ-line expression of an oncogenic erbB2 allele confers resistance to erbB2-induced mammary tumorigenesis. *Proc Natl Acad Sci U S A*.

Andrulis, I. L., Bull, S. B., Blackstein, M. E., Sutherland, D., Mak, C., Sidlofsky, S., Pritzker, K. P., Hartwick, R. W., Hanna, W., Lickley, L. et al. (1998). neu/erbB-2 amplification identifies a poor-prognosis group of women with node-negative breast cancer. Toronto Breast Cancer Study Group. *J Clin Oncol* **16**, 1340-9.

Atwal, J. K., Massie, B., Miller, F. D. and Kaplan, D. R. (2000). The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. *Neuron* **27**, 265-77.

Bao, J., Gur, G. and Yarden, Y. (2003). Src promotes destruction of c-Cbl: implications for oncogenic synergy between Src and growth factor receptors. *Proc Natl Acad Sci U S A* **100**, 2438-43.

Bargmann, C. I., Hung, M. C. and Weinberg, R. A. (1986a). Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell* **45**, 649-57.

Bargmann, C. I., Hung, M. C. and Weinberg, R. A. (1986b). The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature* **319**, 226-30.

Bargmann, C. I. and Weinberg, R. A. (1988). Increased tyrosine kinase activity associated with the protein encoded by the activated neu oncogene. *Proc Natl Acad Sci U S A* **85**, 5394-8.

Batzer, A. G., Rotin, D., Urena, J. M., Skolnik, E. Y. and Schlessinger, J. (1994). Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. *Mol Cell Biol* **14**, 5192-201.

Beerli, R. R., Wels, W. and Hynes, N. E. (1994). Intracellular expression of single chain antibodies reverts ErbB-2 transformation. *J Biol Chem* **269**, 23931-6.

Ben-Levy, R., Paterson, H. F., Marshall, C. J. and Yarden, Y. (1994). A single autophosphorylation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway. *Embo J* **13**, 3302-11.

Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V. and Margolis, B. (1994). A region in Shc distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors. *J Biol Chem* **269**, 32031-4.

Borg, J. P., Ooi, J., Levy, E. and Margolis, B. (1996). The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. *Mol Cell Biol* **16**, 6229-41.

Brennan, P. J., Kumagai, T., Berezov, A., Murali, R., Greene, M. I. and Kumogai, T. (2000). HER2/neu: mechanisms of dimerization/oligomerization. *Oncogene* **19**, 6093-101.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C. and Riethmacher, D. (1998). The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes Dev* **12**, 1825-36.

Buday, L., Khwaja, A., Sipeki, S., Farago, A. and Downward, J. (1996). Interactions of Cbl with two adapter proteins, Grb2 and Crk, upon T cell activation. *J Biol Chem* **271**, 6159-63.

Burden, S. and Yarden, Y. (1997). Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron* **18**, 847-55.

Burgess, A. W., Cho, H. S., Eigenbrot, C., Ferguson, K. M., Garrett, T. P., Leahy, D. J., Lemmon, M. A., Sliwkowski, M. X., Ward, C. W. and Yokoyama, S. (2003). An

open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. *Mol Cell* **12**, 541-52.

Carpenter, G., King, L., Jr. and Cohen, S. (1978). Epidermal growth factor stimulates phosphorylation in membrane preparations in vitro. *Nature* **276**, 409-10.

Carraway, K. L., 3rd and Cantley, L. C. (1994). A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. *Cell* **78**, 5-8.

Carraway, K. L., 3rd, Rossi, E. A., Komatsu, M., Price-Schiavi, S. A., Huang, D., Guy, P. M., Carvajal, M. E., Fregien, N., Carraway, C. A. and Carraway, K. L. (1999). An intramembrane modulator of the ErbB2 receptor tyrosine kinase that potentiates neuregulin signaling. *J Biol Chem* **274**, 5263-6.

Carraway, K. L., 3rd, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C. and Cerione, R. A. (1994). The erbB3 gene product is a receptor for heregulin. *J Biol Chem* **269**, 14303-6.

Carraway, K. L., 3rd, Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Gassmann, M. and Lai, C. (1997). Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. *Nature* **387**, 512-6.

Chan, R., Hardy, W. R., Laing, M. A., Hardy, S. E. and Muller, W. J. (2002). The catalytic activity of the ErbB-2 receptor tyrosine kinase is essential for embryonic development. *Mol Cell Biol* **22**, 1073-8.

Chan, R., Muller, W. J. and Siegel, P. M. (1999). Oncogenic activating mutations in the neu/erbB-2 oncogene are involved in the induction of mammary tumors. *Ann N Y Acad Sci* **889**, 45-51.

Chang, H., Riese, D. J., 2nd, Gilbert, W., Stern, D. F. and McMahan, U. J. (1997). Ligands for ErbB-family receptors encoded by a neuregulin-like gene. *Nature* **387**, 509-12.

Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K. and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **243**, 1576-83.

Chen, W. S., Lazar, C. S., Lund, K. A., Welsh, J. B., Chang, C. P., Walton, G. M., Der, C. J., Wiley, H. S., Gill, G. N. and Rosenfeld, M. G. (1989). Functional independence of the epidermal growth factor receptor from a domain required for ligand-induced internalization and calcium regulation. *Cell* **59**, 33-43.

Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N. and Rosenfeld, M. G. (1987). Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature* **328**, 820-3.

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

Cho, H. S., Mason, K., Ramyar, K. X., Stanley, A. M., Gabelli, S. B., Denney, D. W., Jr. and Leahy, D. J. (2003). Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* **421**, 756-60.

Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A. and Rosen, O. M. (1987). Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. *J Biol Chem* **262**, 1842-7.

Cohen, B. D., Kiener, P. A., Green, J. M., Foy, L., Fell, H. P. and Zhang, K. (1996). The relationship between human epidermal growth-like factor receptor expression and cellular transformation in NIH3T3 cells. *J Biol Chem* **271**, 30897-903.

Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U. et al. (1985). Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* **230**, 1132-9.

Crone, S. A., Zhao, Y. Y., Fan, L., Gu, Y., Minamisawa, S., Liu, Y., Peterson, K. L., Chen, J., Kahn, R., Condorelli, G. et al. (2002). ErbB2 is essential in the prevention of dilated cardiomyopathy. *Nat Med* **8**, 459-65.

Dankort, D., Jeyabalan, N., Jones, N., Dumont, D. J. and Muller, W. J. (2001a). Multiple ErbB-2/Neu Phosphorylation Sites Mediate Transformation through Distinct Effector Proteins. *J Biol Chem* **276**, 38921-8.

Dankort, D., Maslikowski, B., Warner, N., Kanno, N., Kim, H., Wang, Z., Moran, M. F., Oshima, R. G., Cardiff, R. D. and Muller, W. J. (2001b). Grb2 and Shc adapter proteins play distinct roles in Neu (ErbB-2)-induced mammary tumorigenesis: implications for human breast cancer. *Mol Cell Biol* **21**, 1540-51.

Dankort, D. L. and Muller, W. J. (2000). Signal transduction in mammary tumorigenesis: a transgenic perspective. *Oncogene* **19**, 1038-44.

Dankort, D. L., Wang, Z., Blackmore, V., Moran, M. F. and Muller, W. J. (1997). Distinct tyrosine autophosphorylation sites negatively and positively modulate neu-mediated transformation. *Mol Cell Biol* **17**, 5410-25.

de Beer, T., Carter, R. E., Lobel-Rice, K. E., Sorkin, A. and Overduin, M. (1998). Structure and Asn-Pro-Phe binding pocket of the Eps15 homology domain. *Science* **281**, 1357-60.

de Melker, A. A., van der Horst, G., Calafat, J., Jansen, H. and Borst, J. (2001). c-Cbl ubiquitinates the EGF receptor at the plasma membrane and remains receptor associated throughout the endocytic route. *J Cell Sci* **114**, 2167-78.

Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994). A 26 S protease subunit that binds ubiquitin conjugates. *J Biol Chem* **269**, 7059-61.

Dhalluin, C., Yan, K., Plotnikova, O., Lee, K. W., Zeng, L., Kuti, M., Mujtaba, S., Goldfarb, M. P. and Zhou, M. M. (2000). Structural basis of SNT PTB domain interactions with distinct neurotrophic receptors. *Mol Cell* **6**, 921-9.

Di Fiore, P. P., Segatto, O., Lonardo, F., Fazioli, F., Pierce, J. H. and Aaronson, S. A. (1990). The carboxy-terminal domains of erbB-2 and epidermal growth factor receptor exert different regulatory effects on intrinsic receptor tyrosine kinase function and transforming activity. *Mol Cell Biol* **10**, 2749-56.

Diamonti, A. J., Guy, P. M., Ivanof, C., Wong, K., Sweeney, C. and Carraway, K. L., 3rd. (2002). An RBCC protein implicated in maintenance of steady-state neuregulin receptor levels. *Proc Natl Acad Sci U S A* **99**, 2866-71.

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R. and Jessen, K. R. (1995). Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. *Neuron* **15**, 585-96.

Donovan, J. A., Ota, Y., Langdon, W. Y. and Samelson, L. E. (1996). Regulation of the association of p120cbl with Grb2 in Jurkat T cells. *J Biol Chem* **271**, 26369-74.

Dougall, W. C., Qian, X., Miller, M. J. and Greene, M. I. (1996). Association of signaling proteins with a nonmitogenic heterodimeric complex composed of epidermal growth factor receptor and kinase-inactive p185c-neu. *DNA Cell Biol* **15**, 31-40.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M. D. (1984). Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature* **307**, 521-7.

Earp, H. S., Dawson, T. L., Li, X. and Yu, H. (1995). Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. *Breast Cancer Res Treat* **35**, 115-32.

Eck, M. J., Shoelson, S. E. and Harrison, S. C. (1993). Recognition of a high-affinity phosphotyrosyl peptide by the Src homology-2 domain of p56lck. *Nature* **362**, 87-91.

Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M. and Weinberg, R. A. (1993). Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363**, 45-51.

Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. and Rutter, W. J. (1986). Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell* **45**, 721-32.

Eppenberger-Castori, S., Kueng, W., Benz, C., Caduff, R., Varga, Z., Bannwart, F., Fink, D., Dieterich, H., Hohl, M., Muller, H. et al. (2001). Prognostic and predictive significance of ErbB-2 breast tumor levels measured by enzyme immunoassay. *J Clin Oncol* **19**, 645-56.

Erickson, S. L., O'Shea, K. S., Ghaboosi, N., Loverro, L., Frantz, G., Bauer, M., Lu, L. H. and Moore, M. W. (1997). ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Development* **124**, 4999-5011.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S. and Fischbach, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. *Cell* **72**, 801-15.

Fazioli, F., Kim, U. H., Rhee, S. G., Molloy, C. J., Segatto, O. and Di Fiore, P. P. (1991). The erbB-2 mitogenic signaling pathway: tyrosine phosphorylation of phospholipase C-gamma and GTPase-activating protein does not correlate with erbB-2 mitogenic potency. *Mol Cell Biol* **11**, 2040-8.

Fedorov, A. A., Fedorov, E., Gertler, F. and Almo, S. C. (1999). Structure of EVH1, a novel proline-rich ligand-binding module involved in cytoskeletal dynamics and neural function. *Nat Struct Biol* **6**, 661-5.

Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlessinger, J. and Hopkins, C. R. (1990). Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. *Cell* **61**, 623-34.

Ferguson, K. M., Berger, M. B., Mendrola, J. M., Cho, H. S., Leahy, D. J. and Lemmon, M. A. (2003). EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. *Mol Cell* **11**, 507-17.

Feshchenko, E. A., Langdon, W. Y. and Tsygankov, A. Y. (1998). Fyn, Yes, and Syk phosphorylation sites in c-Cbl map to the same tyrosine residues that become phosphorylated in activated T cells. *J Biol Chem* **273**, 8323-31.

Fleishman, S. J., Schlessinger, J. and Ben-Tal, N. (2002). A putative molecular-activation switch in the transmembrane domain of erbB2. *Proc Natl Acad Sci U S A* **99**, 15937-40.

Ford, B. D., Loeb, J. A. and Fischbach, G. D. (1999). Neuregulin stimulates DNA synthesis in embryonic chick heart cells. *Dev Biol* **214**, 139-50.

Fournier, T. M., Lamorte, L., Maroun, C. R., Lupher, M., Band, H., Langdon, W. and Park, M. (2000). Cbl-transforming variants trigger a cascade of molecular alterations that lead to epithelial mesenchymal conversion. *Mol Biol Cell* **11**, 3397-410.

Fowler, K. J., Walker, F., Alexander, W., Hibbs, M. L., Nice, E. C., Bohmer, R. M., Mann, G. B., Thumwood, C., Maglitto, R., Danks, J. A. et al. (1995). A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. *Proc Natl Acad Sci U S A* **92**, 1465-9.

Fukazawa, T., Miyake, S., Band, V. and Band, H. (1996). Tyrosine phosphorylation of Cbl upon epidermal growth factor (EGF) stimulation and its association with EGF receptor and downstream signaling proteins. *J Biol Chem* **271**, 14554-9.

Fukazawa, T., Reedquist, K. A., Panchamoorthy, G., Soltoff, S., Trub, T., Druker, B., Cantley, L., Shoelson, S. E. and Band, H. (1995). T cell activation-dependent association between the p85 subunit of the phosphatidylinositol 3-kinase and Grb2/phospholipase C-gamma 1-binding phosphotyrosyl protein pp36/38. *J Biol Chem* **270**, 20177-82.

Futter, C. E., Pearse, A., Hewlett, L. J. and Hopkins, C. R. (1996). Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. *J Cell Biol* **132**, 1011-23.

Galan, J. M. and Haguenauer-Tsapis, R. (1997). Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. *Embo J* **16**, 5847-54.

Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlessinger, J. and Bar-Sagi, D. (1993). Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. *Nature* **363**, 88-92.

Garcia-Guzman, M., Larsen, E. and Vuori, K. (2000). The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: a role for the adaptor protein Crk. *Oncogene* **19**, 4058-65.

Garratt, A. N., Britsch, S. and Birchmeier, C. (2000a). Neuregulin, a factor with many functions in the life of a schwann cell. *Bioessays* **22**, 987-96.

Garratt, A. N., Voiculescu, O., Topilko, P., Charnay, P. and Birchmeier, C. (2000b). A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. *J Cell Biol* **148**, 1035-46.

Garrett, T. P., McKern, N. M., Lou, M., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Kofler, M., Jorissen, R. N., Nice, E. C., Burgess, A. W. et al. (2003). The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Mol Cell* **11**, 495-505.

Garrett, T. P., McKern, N. M., Lou, M., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Zhu, H. J., Walker, F., Frenkel, M. J., Hoyne, P. A. et al. (2002). Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. *Cell* **110**, 763-73.

Gasparini, G., Gullick, W. J., Maluta, S., Dalla Palma, P., Caffo, O., Leonardi, E., Boracchi, P., Pozza, F., Lemoine, N. R. and Bevilacqua, P. (1994). c-erbB-3 and c-erbB-2 protein expression in node-negative breast carcinoma--an immunocytochemical study. *Eur J Cancer* **30A**, 16-22.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-4.

Gilboa, L., Ben-Levy, R., Yarden, Y. and Henis, Y. I. (1995). Roles for a cytoplasmic tyrosine and tyrosine kinase activity in the interactions of Neu receptors with coated pits. *J Biol Chem* **270**, 7061-7.

Goldman, R., Levy, R. B., Peles, E. and Yarden, Y. (1990). Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. *Biochemistry* **29**, 11024-8.

Gorden, P., Carpentier, J. L., Cohen, S. and Orci, L. (1978). Epidermal growth factor: morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. *Proc Natl Acad Sci U S A* **75**, 5025-9.

Gotoh, N., Tojo, A., Hino, M., Yazaki, Y. and Shibuya, M. (1992). A highly conserved tyrosine residue at codon 845 within the kinase domain is not required for the transforming activity of human epidermal growth factor receptor. *Biochem Biophys Res Commun* **186**, 768-74.

Graus-Porta, D., Beerli, R. R., Daly, J. M. and Hynes, N. E. (1997). ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *Embo J* **16**, 1647-55.

Graus-Porta, D., Beerli, R. R. and Hynes, N. E. (1995). Single-chain antibody-mediated intracellular retention of ErbB-2 impairs Neu differentiation factor and epidermal growth factor signaling. *Mol Cell Biol* **15**, 1182-91.

Gu, H., Maeda, H., Moon, J. J., Lord, J. D., Yoakim, M., Nelson, B. H. and Neel, B. G. (2000). New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. *Mol Cell Biol* **20**, 7109-20.

Gullapalli, A., Garrett, T. A., Paing, M. M., Griffin, C. T., Yang, Y. and Trejo, J. (2004). A Role for Sorting Nexin 2 in Epidermal Growth Factor Receptor Down-regulation: Evidence for Distinct Functions of Sorting Nexin 1 and 2 in Protein Trafficking. *Mol Biol Cell.*

Gullick, W. J. (1994). A new model for the interaction of EGF-like ligands with their receptors: the new one-two. *Eur J Cancer* **30A**, 2186.

Gullick, W. J., Love, S. B., Wright, C., Barnes, D. M., Gusterson, B., Harris, A. L. and Altman, D. G. (1991). c-erbB-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. *Br J Cancer* **63**, 434-8.

Guy, C. T., Muthuswamy, S. K., Cardiff, R. D., Soriano, P. and Muller, W. J. (1994a). Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice. *Genes Dev* **8**, 23-32.

Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D. and Muller, W. J. (1992). Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A* **89**, 10578-82.

Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A. and Carraway, K. L., 3rd. (1994b). Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci U S A* **91**, 8132-6.

Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P. and Dikic, I. (2003). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat Cell Biol* **5**, 461-6.

Haigler, H. T., McKanna, J. A. and Cohen, S. (1979). Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. *J Cell Biol* **81**, 382-95.

Harari, D., Tzahar, E., Romano, J., Shelly, M., Pierce, J. H., Andrews, G. C. and Yarden, Y. (1999). Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. *Oncogene* **18**, 2681-9.

Harris, A. L., Nicholson, S., Sainsbury, J. R., Farndon, J. and Wright, C. (1989). Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. *J Steroid Biochem* **34**, 123-31.

Hazan, R., Margolis, B., Dombalagian, M., Ullrich, A., Zilberstein, A. and Schlessinger, J. (1990). Identification of autophosphorylation sites of HER2/neu. *Cell Growth Differ* **1**, 3-7.

Hempstead, B. L., Birge, R. B., Fajardo, J. E., Glassman, R., Mahadeo, D., Kraemer, R. and Hanafusa, H. (1994). Expression of the v-crk oncogene product in PC12 cells results in rapid differentiation by both nerve growth factor- and epidermal growth factor-dependent pathways. *Mol Cell Biol* **14**, 1964-71.

Hertig, C. M., Kubalak, S. W., Wang, Y. and Chien, K. R. (1999). Synergistic roles of neuregulin-1 and insulin-like growth factor-I in activation of the phosphatidylinositol 3-kinase pathway and cardiac chamber morphogenesis. *J Biol Chem* **274**, 37362-9.

Hicke, L. (2001). Protein regulation by monoubiquitin. *Nat Rev Mol Cell Biol* **2**, 195-201.

Hicke, L., Zanolari, B. and Riezman, H. (1998). Cytoplasmic tail phosphorylation of the alpha-factor receptor is required for its ubiquitination and internalization. *J Cell Biol* **141**, 349-58.

Hime, G. R., Dhungat, M. P., Ng, A. and Bowtell, D. D. (1997). D-Cbl, the Drosophila homologue of the c-Cbl proto-oncogene, interacts with the Drosophila EGF receptor in vivo, despite lacking C-terminal adaptor binding sites. *Oncogene* **14**, 2709-19.

Hislop, J. N., Marley, A. and Von Zastrow, M. (2004). Role of mammalian VPS proteins in endocytic trafficking of a non-ubiquitinated G protein-coupled receptor to lysosomes. *J Biol Chem.*

Hofmann, R. M. and Pickart, C. M. (1999). Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell* **96**, 645-53.

Holbro, T., Beerli, R. R., Maurer, F., Koziczak, M., Barbas, C. F., 3rd and Hynes, N. E. (2003). The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci U S A* **100**, 8933-8.

Holbrook, M. R., O'Donnell, J. B., Jr., Slakey, L. L. and Gross, D. J. (1999). Epidermal growth factor receptor internalization rate is regulated by negative charges near the SH2 binding site Tyr992. *Biochemistry* **38**, 9348-56.

Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D. et al. (1992). Identification of heregulin, a specific activator of p185erbB2. *Science* **256**, 1205-10.

Honegger, A. M., Dull, T. J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A. and Schlessinger, J. (1987). Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. *Cell* **51**, 199-209.

Hubbard, S. R., Mohammadi, M. and Schlessinger, J. (1998). Autoregulatory mechanisms in protein-tyrosine kinases. *J Biol Chem* **273**, 11987-90.

Hubbard, S. R. and Till, J. H. (2000). Protein tyrosine kinase structure and function. *Annu Rev Biochem* **69**, 373-98.

Hunter, T. and Sefton, B. M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc Natl Acad Sci U S A* **77**, 1311-5.

Hurley, J. H. and Meyer, T. (2001). Subcellular targeting by membrane lipids. *Curr Opin Cell Biol* **13**, 146-52.

Huse, M. and Kuriyan, J. (2002). The conformational plasticity of protein kinases. *Cell* **109**, 275-82.

Hynes, N. E. and Stern, D. F. (1994). The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta* **1198**, 165-84.

Jallal, B., Schlessinger, J. and Ullrich, A. (1992). Tyrosine phosphatase inhibition permits analysis of signal transduction complexes in p185HER2/neu-overexpressing human tumor cells. *J Biol Chem* **267**, 4357-63.

Janes, P. W., Daly, R. J., deFazio, A. and Sutherland, R. L. (1994). Activation of the Ras signalling pathway in human breast cancer cells overexpressing erbB-2. *Oncogene* **9**, 3601-8.

Jepson, S., Komatsu, M., Haq, B., Arango, M. E., Huang, D., Carraway, C. A. and Carraway, K. L. (2002). Muc4/sialomucin complex, the intramembrane ErbB2 ligand, induces specific phosphorylation of ErbB2 and enhances expression of p27(kip), but does not activate mitogen-activated kinase or protein kinaseB/Akt pathways. *Oncogene* **21**, 7524-32.

Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T. and Liu, Y. C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **286**, 309-12.

Karunagaran, D., Tzahar, E., Beerli, R. R., Chen, X., Graus-Porta, D., Ratzkin, B. J., Seger, R., Hynes, N. E. and Yarden, Y. (1996). ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *Embo J* **15**, 254-64.

Katzmann, D. J., Babst, M. and Emr, S. D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell* **106**, 145-55.

Katzmann, D. J., Odorizzi, G. and Emr, S. D. (2002). Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol* **3**, 893-905.

Kavanaugh, W. M., Turck, C. W. and Williams, L. T. (1995). PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science* **268**, 1177-9.

Kavanaugh, W. M. and Williams, L. T. (1994). An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science* **266**, 1862-5.

Keane, M. M., Ettenberg, S. A., Nau, M. M., Banerjee, P., Cuello, M., Penninger, J. and Lipkowitz, S. (1999). cbl-3: a new mammalian cbl family protein. *Oncogene* **18**, 3365-75.

Keane, M. M., Rivero-Lezcano, O. M., Mitchell, J. A., Robbins, K. C. and Lipkowitz, S. (1995). Cloning and characterization of cbl-b: a SH3 binding protein with homology to the c-cbl proto-oncogene. *Oncogene* **10**, 2367-77.

King, C. R., Borrello, I., Bellot, F., Comoglio, P. and Schlessinger, J. (1988). Egf binding to its receptor triggers a rapid tyrosine phosphorylation of the erbB-2 protein in the mammary tumor cell line SK-BR-3. *Embo J* **7**, 1647-51.

Klapper, L. N., Waterman, H., Sela, M. and Yarden, Y. (2000). Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. *Cancer Res* **60**, 3384-8.

Kramer, R., Bucay, N., Kane, D. J., Martin, L. E., Tarpley, J. E. and Theill, L. E. (1996). Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc Natl Acad Sci U S A* **93**, 4833-8.

Kraus, M. H., Issing, W., Miki, T., Popescu, N. C. and Aaronson, S. A. (1989). Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. *Proc Natl Acad Sci U S A* **86**, 9193-7.

Kurten, R. C., Cadena, D. L. and Gill, G. N. (1996). Enhanced degradation of EGF receptors by a sorting nexin, SNX1. *Science* **272**, 1008-10.

Kwong, K. Y. and Hung, M. C. (1998). A novel splice variant of HER2 with increased transformation activity. *Mol Carcinog* **23**, 62-8.

Lai, K. M. and Pawson, T. (2000). The ShcA phosphotyrosine docking protein sensitizes cardiovascular signaling in the mouse embryo. *Genes Dev* **14**, 1132-45.

Langdon, W. Y., Hartley, J. W., Klinken, S. P., Ruscetti, S. K. and Morse, H. C., 3rd. (1989). v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. *Proc Natl Acad Sci U S A* **86**, 1168-72.

Lax, I., Bellot, F., Howk, R., Ullrich, A., Givol, D. and Schlessinger, J. (1989). Functional analysis of the ligand binding site of EGF-receptor utilizing chimeric chicken/human receptor molecules. *Embo J* **8**, 421-7.

Le Douarin, N. M. (1986). Cell line segregation during peripheral nervous system ontogeny. *Science* **231**, 1515-22.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C. and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-8.

Lee, P. S., Wang, Y., Dominguez, M. G., Yeung, Y. G., Murphy, M. A., Bowtell, D. D. and Stanley, E. R. (1999). The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. *Embo J* **18**, 3616-28.

Lemmon, M. A., Bu, Z., Ladbury, J. E., Zhou, M., Pinchasi, D., Lax, I., Engelman, D. M. and Schlessinger, J. (1997). Two EGF molecules contribute additively to stabilization of the EGFR dimer. *Embo J* **16**, 281-94.

Lemoine, N. R., Barnes, D. M., Hollywood, D. P., Hughes, C. M., Smith, P., Dublin, E., Prigent, S. A., Gullick, W. J. and Hurst, H. C. (1992). Expression of the ERBB3 gene product in breast cancer. *Br J Cancer* **66**, 1116-21.

Lemoine, N. R., Staddon, S., Dickson, C., Barnes, D. M. and Gullick, W. J. (1990). Absence of activating transmembrane mutations in the c-erbB-2 proto-oncogene in human breast cancer. *Oncogene* **5**, 237-9.

Levi, A. D., Bunge, R. P., Lofgren, J. A., Meima, L., Hefti, F., Nikolics, K. and Sliwkowski, M. X. (1995). The influence of heregulins on human Schwann cell proliferation. *J Neurosci* **15**, 1329-40.

Levkowitz, G., Oved, S., Klapper, L. N., Harari, D., Lavi, S., Sela, M. and Yarden, Y. (2000). c-Cbl is a suppressor of the neu oncogene. *J Biol Chem* **275**, 35532-9.

Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A. et al. (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol Cell* **4**, 1029-40.

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B. and Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* **12**, 3663-74.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B. and Schlessinger, J. (1993). Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature* **363**, 85-8.

Lill, N. L., Douillard, P., Awwad, R. A., Ota, S., Lupher, M. L., Jr., Miyake, S., Meissner-Lula, N., Hsu, V. W. and Band, H. (2000). The evolutionarily conserved N-terminal region of Cbl is sufficient to enhance down-regulation of the epidermal growth factor receptor. *J Biol Chem* **275**, 367-77.

Link, A. J., Eng, J., Schieltz, D. M., Carmack, E., Mize, G. J., Morris, D. R., Garvik, B. M. and Yates, J. R., 3rd. (1999). Direct analysis of protein complexes using mass spectrometry. *Nat Biotechnol* **17**, 676-82.

Liu, X., Hwang, H., Cao, L., Buckland, M., Cunningham, A., Chen, J., Chien, K. R., Graham, R. M. and Zhou, M. (1998). Domain-specific gene disruption reveals critical regulation of neuregulin signaling by its cytoplasmic tail. *Proc Natl Acad Sci U S A* **95**, 13024-9.

Longva, K. E., Blystad, F. D., Stang, E., Larsen, A. M., Johannessen, L. E. and Madshus, I. H. (2002). Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. *J Cell Biol* **156**, 843-54.

Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-Sagi, D. and Schlessinger, J. (1992). The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* **70**, 431-42.

Luetteke, N. C., Phillips, H. K., Qiu, T. H., Copeland, N. G., Earp, H. S., Jenkins, N. A. and Lee, D. C. (1994). The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. *Genes Dev* **8**, 399-413.

Lupher, M. L., Jr., Reedquist, K. A., Miyake, S., Langdon, W. Y. and Band, H. (1996). A novel phosphotyrosine-binding domain in the N-terminal transforming region of Cbl interacts directly and selectively with ZAP-70 in T cells. *J Biol Chem* **271**, 24063-8.

Luttrell, D. K., Lee, A., Lansing, T. J., Crosby, R. M., Jung, K. D., Willard, D., Luther, M., Rodriguez, M., Berman, J. and Gilmer, T. M. (1994). Involvement of pp60c-src with two major signaling pathways in human breast cancer. *Proc Natl Acad Sci U S A* **91**, 83-7.

Luttrell, D. K., Luttrell, L. M. and Parsons, S. J. (1988). Augmented mitogenic responsiveness to epidermal growth factor in murine fibroblasts that overexpress pp60c-src. *Mol Cell Biol* **8**, 497-501.

Maa, M. C., Leu, T. H., McCarley, D. J., Schatzman, R. C. and Parsons, S. J. (1995). Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: implications for the etiology of multiple human cancers. *Proc Natl Acad Sci U S A* **92**, 6981-5.

Macias, M. J., Hyvonen, M., Baraldi, E., Schultz, J., Sudol, M., Saraste, M. and Oschkinat, H. (1996). Structure of the WW domain of a kinase-associated protein complexed with a proline-rich peptide. *Nature* **382**, 646-9.

Maina, F., Casagranda, F., Audero, E., Simeone, A., Comoglio, P. M., Klein, R. and Ponzetto, C. (1996). Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. *Cell* **87**, 531-42.

Mann, G. B., Fowler, K. J., Gabriel, A., Nice, E. C., Williams, R. L. and Dunn, A. R. (1993). Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* **73**, 249-61.

Marchionni, M. A., Goodearl, A. D., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K. et al. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* **362**, 312-8.

Marengere, L. E., Songyang, Z., Gish, G. D., Schaller, M. D., Parsons, J. T., Stern, M. J., Cantley, L. C. and Pawson, T. (1994). SH2 domain specificity and activity modified by a single residue. *Nature* **369**, 502-5.

Margolis, B. L., Lax, I., Kris, R., Dombalagian, M., Honegger, A. M., Howk, R., Givol, D., Ullrich, A. and Schlessinger, J. (1989). All autophosphorylation sites of epidermal growth factor (EGF) receptor and HER2/neu are located in their carboxyl-terminal tails. Identification of a novel site in EGF receptor. *J Biol Chem* **264**, 10667-71.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179-85.

Mayer, B. J. (2001). SH3 domains: complexity in moderation. *J Cell Sci* **114**, 1253-63.

McClain, D. A., Maegawa, H., Lee, J., Dull, T. J., Ulrich, A. and Olefsky, J. M. (1987). A mutant insulin receptor with defective tyrosine kinase displays no biologic activity and does not undergo endocytosis. *J Biol Chem* **262**, 14663-71.

McCormick, F. (1994). Activators and effectors of ras p21 proteins. *Curr Opin Genet Dev* **4**, 71-6.

McGlade, J., Cheng, A., Pelicci, G., Pelicci, P. G. and Pawson, T. (1992). Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases. *Proc Natl Acad Sci U S A* **89**, 8869-73.

Meisner, H., Conway, B. R., Hartley, D. and Czech, M. P. (1995). Interactions of Cbl with Grb2 and phosphatidylinositol 3'-kinase in activated Jurkat cells. *Mol Cell Biol* **15**, 3571-8.

Meisner, H. and Czech, M. P. (1995). Coupling of the proto-oncogene product c-Cbl to the epidermal growth factor receptor. *J Biol Chem* **270**, 25332-5.

Meisner, H., Daga, A., Buxton, J., Fernandez, B., Chawla, A., Banerjee, U. and Czech, M. P. (1997). Interactions of Drosophila Cbl with epidermal growth factor receptors and role of Cbl in R7 photoreceptor cell development. *Mol Cell Biol* **17**, 2217-25.

Meng, W., Sawasdikosol, S., Burakoff, S. J. and Eck, M. J. (1999). Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase. *Nature* **398**, 84-90.

Meyer, D. and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378**, 386-90.

Mikami, Y., Davis, J. G., Dobashi, K., Dougall, W. C., Myers, J. N., Brown, V. I. and Greene, M. I. (1992). Carboxyl-terminal deletion and point mutations decrease the transforming potential of the activated rat neu oncogene product. *Proc Natl Acad Sci U S A* **89**, 7335-9.

Miller, K., Beardmore, J., Kanety, H., Schlessinger, J. and Hopkins, C. R. (1986). Localization of the epidermal growth factor (EGF) receptor within the endosome of EGF-stimulated epidermoid carcinoma (A431) cells. *J Cell Biol* **102**, 500-9.

Miyake, S., Mullane-Robinson, K. P., Lill, N. L., Douillard, P. and Band, H. (1999). Cbl-mediated negative regulation of platelet-derived growth factor receptor-dependent cell proliferation. A critical role for Cbl tyrosine kinase-binding domain. *J Biol Chem* **274**, 16619-28.

Mohammadi, M., Dikic, I., Sorokin, A., Burgess, W. H., Jaye, M. and Schlessinger, J. (1996). Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. *Mol Cell Biol* **16**, 977-89.

Morais Cabral, J. H., Petosa, C., Sutcliffe, M. J., Raza, S., Byron, O., Poy, F., Marfatia, S. M., Chishti, A. H. and Liddington, R. C. (1996). Crystal structure of a PDZ domain. *Nature* **382**, 649-52.

Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D. and Lee, K. F. (1999). Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. *Neuron* **23**, 273-83.

Mosesson, Y., Shtiegman, K., Katz, M., Zwang, Y., Vereb, G., Szollosi, J. and Yarden, Y. (2003). Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. *J Biol Chem* **278**, 21323-6.

Murphy, M. A., Schnall, R. G., Venter, D. J., Barnett, L., Bertoncello, I., Thien, C. B., Langdon, W. Y. and Bowtell, D. D. (1998). Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. *Mol Cell Biol* **18**, 4872-82.

Muthuswamy, S. K., Gilman, M. and Brugge, J. S. (1999). Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. *Mol Cell Biol* **19**, 6845-57.

Muthuswamy, S. K. and Muller, W. J. (1995a). Activation of Src family kinases in Neu-induced mammary tumors correlates with their association with distinct sets of tyrosine phosphorylated proteins in vivo. *Oncogene* **11**, 1801-10.

Muthuswamy, S. K. and Muller, W. J. (1995b). Direct and specific interaction of c-Src with Neu is involved in signaling by the epidermal growth factor receptor. *Oncogene* **11**, 271-9.

Muthuswamy, S. K., Siegel, P. M., Dankort, D. L., Webster, M. A. and Muller, W. J. (1994). Mammary tumors expressing the neu proto-oncogene possess elevated c-Src tyrosine kinase activity. *Mol Cell Biol* **14**, 735-43.

Nagy, A., Moens, C., Ivanyi, E., Pawling, J., Gertsenstein, M., Hadjantonakis, A. K., Pirity, M. and Rossant, J. (1998). Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. *Curr Biol* **8**, 661-4.

Nakatsu, F., Sakuma, M., Matsuo, Y., Arase, H., Yamasaki, S., Nakamura, N., Saito, T. and Ohno, H. (2000). A Di-leucine signal in the ubiquitin moiety. Possible involvement in ubiquitination-mediated endocytosis. *J Biol Chem* **275**, 26213-9.

Noonberg, S. B. and Benz, C. C. (2000). Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily: role as anticancer agents. *Drugs* **59**, 753-67.

Normanno, N., Ciardiello, F., Brandt, R. and Salomon, D. S. (1994). Epidermal growth factor-related peptides in the pathogenesis of human breast cancer. *Breast Cancer Res Treat* **29**, 11-27.

Ogiso, H., Ishitani, R., Nureki, O., Fukai, S., Yamanaka, M., Kim, J. H., Saito, K., Sakamoto, A., Inoue, M., Shirouzu, M. et al. (2002). Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. *Cell* **110**, 775-87.

Okutani, T., Okabayashi, Y., Kido, Y., Sugimoto, Y., Sakaguchi, K., Matuoka, K., Takenawa, T. and Kasuga, M. (1994). Grb2/Ash binds directly to tyrosines 1068 and 1086 and indirectly to tyrosine 1148 of activated human epidermal growth factor receptors in intact cells. *J Biol Chem* **269**, 31310-4.

Olayioye, M. A., Neve, R. M., Lane, H. A. and Hynes, N. E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. *Embo J* **19**, 3159-67.

Ong, S. H., Guy, G. R., Hadari, Y. R., Laks, S., Gotoh, N., Schlessinger, J. and Lax, I. (2000). FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. *Mol Cell Biol* **20**, 979-89.

Ozcelik, C., Erdmann, B., Pilz, B., Wettschureck, N., Britsch, S., Hubner, N., Chien, K. R., Birchmeier, C. and Garratt, A. N. (2002). Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proc Natl Acad Sci U S A* **99**, 8880-5.

Padhy, L. C., Shih, C., Cowing, D., Finkelstein, R. and Weinberg, R. A. (1982). Identification of a phosphoprotein specifically induced by the transforming DNA of rat neuroblastomas. *Cell* **28**, 865-71.

Pai, L. M., Barcelo, G. and Schupbach, T. (2000). D-cbl, a negative regulator of the Egfr pathway, is required for dorsoventral patterning in Drosophila oogenesis. *Cell* **103**, 51-61.

Partanen, J., Schwartz, L. and Rossant, J. (1998). Opposite phenotypes of hypomorphic and Y766 phosphorylation site mutations reveal a function for Fgfr1 in anteroposterior patterning of mouse embryos. *Genes Dev* **12**, 2332-44.

Pascal, S. M., Singer, A. U., Gish, G., Yamazaki, T., Shoelson, S. E., Pawson, T., Kay, L. E. and Forman-Kay, J. D. (1994). Nuclear magnetic resonance structure of an

SH2 domain of phospholipase C-gamma 1 complexed with a high affinity binding peptide. *Cell* **77**, 461-72.

Paterson, M. C., Dietrich, K. D., Danyluk, J., Paterson, A. H., Lees, A. W., Jamil, N., Hanson, J., Jenkins, H., Krause, B. E., McBlain, W. A. et al. (1991). Correlation between c-erbB-2 amplification and risk of recurrent disease in node-negative breast cancer. *Cancer Res* **51**, 556-67.

Pawson, T. (1995). Protein modules and signalling networks. *Nature* **373**, 573-80.

Pawson, T. and Nash, P. (2000). Protein-protein interactions define specificity in signal transduction. *Genes Dev* **14**, 1027-47.

Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Levy, R. B. and Yarden, Y. (1992a). Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell* **69**, 205-16.

Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D. and Yarden, Y. (1993). Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests complex ligand-receptor relationships. *Embo J* **12**, 961-71.

Peles, E., Lamprecht, R., Ben-Levy, R., Tzahar, E. and Yarden, Y. (1992b). Regulated coupling of the Neu receptor to phosphatidylinositol 3'-kinase and its release by oncogenic activation. *J Biol Chem* **267**, 12266-74.

Peles, E., Levy, R. B., Or, E., Ullrich, A. and Yarden, Y. (1991). Oncogenic forms of the neu/HER2 tyrosine kinase are permanently coupled to phospholipase C gamma. *Embo J* **10**, 2077-86.

Peles, E. and Yarden, Y. (1993). Neu and its ligands: from an oncogene to neural factors. *Bioessays* **15**, 815-24.

Penengo, L., Rubin, C., Yarden, Y. and Gaudino, G. (2003). c-Cbl is a critical modulator of the Ron tyrosine kinase receptor. *Oncogene* **22**, 3669-79.

Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y. and Park, M. (2001). Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol Cell* **8**, 995-1004.

Peschard, P. and Park, M. (2003). Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases. *Cancer Cell* **3**, 519-23.

Pinkas-Kramarski, R., Lenferink, A. E., Bacus, S. S., Lyass, L., van de Poll, M. L., Klapper, L. N., Tzahar, E., Sela, M., van Zoelen, E. J. and Yarden, Y. (1998a). The

oncogenic ErbB-2/ErbB-3 heterodimer is a surrogate receptor of the epidermal growth factor and betacellulin. *Oncogene* **16**, 1249-58.

Pinkas-Kramarski, R., Shelly, M., Glathe, S., Ratzkin, B. J. and Yarden, Y. (1996a). Neu differentiation factor/neuregulin isoforms activate distinct receptor combinations. *J Biol Chem* **271**, 19029-32.

Pinkas-Kramarski, R., Shelly, M., Guarino, B. C., Wang, L. M., Lyass, L., Alroy, I., Alimandi, M., Kuo, A., Moyer, J. D., Lavi, S. et al. (1998b). ErbB tyrosine kinases and the two neuregulin families constitute a ligand-receptor network. *Mol Cell Biol* **18**, 6090-101.

Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M. et al. (1996b). Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *Embo J* **15**, 2452-67.

Plowman, G. D., Culouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G. and Shoyab, M. (1993). Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. *Proc Natl Acad Sci U S A* **90**, 1746-50.

Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J. and Shoyab, M. (1990). Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc Natl Acad Sci U S A* **87**, 4905-9.

Press, M. F., Pike, M. C., Chazin, V. R., Hung, G., Udove, J. A., Markowicz, M., Danyluk, J., Godolphin, W., Sliwkowski, M., Akita, R. et al. (1993). Her-2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. *Cancer Res* **53**, 4960-70.

Prigent, S. A. and Gullick, W. J. (1994). Identification of c-erbB-3 binding sites for phosphatidylinositol 3'-kinase and SHC using an EGF receptor/c-erbB-3 chimera. *Embo J* **13**, 2831-41.

Qian, X., Dougall, W. C., Hellman, M. E. and Greene, M. I. (1994a). Kinase-deficient neu proteins suppress epidermal growth factor receptor function and abolish cell transformation. *Oncogene* **9**, 1507-14.

Qian, X., LeVea, C. M., Freeman, J. K., Dougall, W. C. and Greene, M. I. (1994b). Heterodimerization of epidermal growth factor receptor and wild-type or kinase-deficient Neu: a mechanism of interreceptor kinase activation and transphosphorylation. *Proc Natl Acad Sci U S A* **91**, 1500-4.

Qiu, X. B. and Goldberg, A. L. (2002). Nrdp1/FLRF is a ubiquitin ligase promoting ubiquitination and degradation of the epidermal growth factor receptor family member, ErbB3. *Proc Natl Acad Sci U S A* **99**, 14843-8.

Quinn, C. M., Ostrowski, J. L., Lane, S. A., Loney, D. P., Teasdale, J. and Benson, F. A. (1994). c-erbB-3 protein expression in human breast cancer: comparison with other tumour variables and survival. *Histopathology* **25**, 247-52.

Raiborg, C., Bache, K. G., Gillooly, D. J., Madshus, I. H., Stang, E. and Stenmark, H. (2002). Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. *Nat Cell Biol* **4**, 394-8.

Ramsauer, V. P., Carraway, C. A., Salas, P. J. and Carraway, K. L. (2003). Muc4/sialomucin complex, the intramembrane ErbB2 ligand, translocates ErbB2 to the apical surface in polarized epithelial cells. *J Biol Chem* **278**, 30142-7.

Ravichandran, K. S. (2001). Signaling via Shc family adapter proteins. *Oncogene* **20**, 6322-30.

Reggiori, F. and Pelham, H. R. (2001). Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. *Embo J* **20**, 5176-86.

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

Riese, D. J., 2nd, van Raaij, T. M., Plowman, G. D., Andrews, G. C. and Stern, D. F. (1995). The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Mol Cell Biol* **15**, 5770-6.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R. and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* **389**, 725-30.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* **17**, 1030-2.

Rivero-Lezcano, O. M., Sameshima, J. H., Marcilla, A. and Robbins, K. C. (1994). Physical association between Src homology 3 elements and the protein product of the c-cbl proto-oncogene. *J Biol Chem* **269**, 17363-6.

Roussel, M. F., Downing, J. R., Rettenmier, C. W. and Sherr, C. J. (1988). A point mutation in the extracellular domain of the human CSF-1 receptor (c-fms proto-oncogene product) activates its transforming potential. *Cell* **55**, 979-88.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993). The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature* **363**, 83-5.

Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G. et al. (1992). Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* **360**, 689-92.

Russell, D. S., Gherzi, R., Johnson, E. L., Chou, C. K. and Rosen, O. M. (1987). The protein-tyrosine kinase activity of the insulin receptor is necessary for insulin-mediated receptor down-regulation. *J Biol Chem* **262**, 11833-40.

Sainsbury, J. R., Farndon, J. R., Needham, G. K., Malcolm, A. J. and Harris, A. L. (1987). Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. *Lancet* **1**, 1398-402.

Schechter, A. L., Stern, D. F., Vaidyanathan, L., Decker, S. J., Drebin, J. A., Greene, M. I. and Weinberg, R. A. (1984). The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. *Nature* **312**, 513-6.

Scott, G. K., Dodson, J. M., Montgomery, P. A., Johnson, R. M., Sarup, J. C., Wong, W. L., Ullrich, A., Shepard, H. M. and Benz, C. C. (1991). p185HER2 signal transduction in breast cancer cells. *J Biol Chem* **266**, 14300-5.

Segatto, O., Lonardo, F., Helin, K., Wexler, D., Fazioli, F., Rhee, S. G. and Di Fiore, P. P. (1992). erbB-2 autophosphorylation is required for mitogenic action and high-affinity substrate coupling. *Oncogene* **7**, 1339-46.

Segatto, O., Pelicci, G., Giuli, S., Digiesi, G., Di Fiore, P. P., McGlade, J., Pawson, T. and Pelicci, P. G. (1993). Shc products are substrates of erbB-2 kinase. *Oncogene* **8**, 2105-12.

Seidman, A., Hudis, C., Pierri, M. K., Shak, S., Paton, V., Ashby, M., Murphy, M., Stewart, S. J. and Keefe, D. (2002). Cardiac dysfunction in the trastuzumab clinical trials experience. *J Clin Oncol* **20**, 1215-21.

Settle, M., Gordon, M. D., Nadella, M., Dankort, D., Muller, W. and Jacobs, J. R. (2003). Genetic identification of effectors downstream of Neu (ErbB-2) autophosphorylation sites in a Drosophila model. *Oncogene* **22**, 1916-26.

Sheng, Z., Wu, K., Carraway, K. L. and Fregien, N. (1992). Molecular cloning of the transmembrane component of the 13762 mammary adenocarcinoma sialomucin complex. A new member of the epidermal growth factor superfamily. *J Biol Chem* **267**, 16341-6.

Shih, C., Padhy, L. C., Murray, M. and Weinberg, R. A. (1981). Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* **290**, 261-4.

Shih, S. C., Katzmann, D. J., Schnell, J. D., Sutanto, M., Emr, S. D. and Hicke, L. (2002). Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. *Nat Cell Biol* **4**, 389-93.

Shih, S. C., Sloper-Mould, K. E. and Hicke, L. (2000). Monoubiquitin carries a novel internalization signal that is appended to activated receptors. *Embo J* **19**, 187-98.

Sibilia, M. and Wagner, E. F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* **269**, 234-8.

Siegel, P. M., Dankort, D. L., Hardy, W. R. and Muller, W. J. (1994). Novel activating mutations in the neu proto-oncogene involved in induction of mammary tumors. *Mol Cell Biol* **14**, 7068-77.

Siegel, P. M. and Muller, W. J. (1996). Mutations affecting conserved cysteine residues within the extracellular domain of Neu promote receptor dimerization and activation. *Proc Natl Acad Sci U S A* **93**, 8878-83.

Siegel, P. M., Ryan, E. D., Cardiff, R. D. and Muller, W. J. (1999). Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. *Embo J* **18**, 2149-64.

Simpson, B. J., Phillips, H. A., Lessells, A. M., Langdon, S. P. and Miller, W. R. (1995). c-erbB growth-factor-receptor proteins in ovarian tumours. *Int J Cancer* **64**, 202-6.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177-82.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**, 707-12.

Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M. et al. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* **344**, 783-92.

Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L. and Carraway, K. L., 3rd.

(1994). Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. *J Biol Chem* **269**, 14661-5.

Songyang, Z., Margolis, B., Chaudhuri, M., Shoelson, S. E. and Cantley, L. C. (1995). The phosphotyrosine interaction domain of SHC recognizes tyrosine-phosphorylated NPXY motif. *J Biol Chem* **270**, 14863-6.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J. et al. (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**, 767-78.

Songyang, Z., Shoelson, S. E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X. R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T. et al. (1994). Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol Cell Biol* **14**, 2777-85.

Sorkin, A., Helin, K., Waters, C. M., Carpenter, G. and Beguinot, L. (1992). Multiple autophosphorylation sites of the epidermal growth factor receptor are essential for receptor kinase activity and internalization. Contrasting significance of tyrosine 992 in the native and truncated receptors. *J Biol Chem* **267**, 8672-8.

Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M. and Finley, D. (2000). Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. *Cell* **102**, 67-76.

Stamos, J., Sliwkowski, M. X. and Eigenbrot, C. (2002). Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. *J Biol Chem* **277**, 46265-72.

Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L. and Rotin, D. (1997). Regulation of stability and function of the epithelial Na+ channel (ENaC) by ubiquitination. *Embo J* **16**, 6325-36.

Stein, D., Wu, J., Fuqua, S. A., Roonprapunt, C., Yajnik, V., D'Eustachio, P., Moskow, J. J., Buchberg, A. M., Osborne, C. K. and Margolis, B. (1994). The SH2 domain protein GRB-7 is co-amplified, overexpressed and in a tight complex with HER2 in breast cancer. *Embo J* **13**, 1331-40.

Stern, D. F. and Kamps, M. P. (1988). EGF-stimulated tyrosine phosphorylation of p185neu: a potential model for receptor interactions. *Embo J* **7**, 995-1001.

Stern, D. F., Kamps, M. P. and Cao, H. (1988). Oncogenic activation of p185neu stimulates tyrosine phosphorylation in vivo. *Mol Cell Biol* **8**, 3969-73.

Sternberg, M. J. and Gullick, W. J. (1989). Neu receptor dimerization. *Nature* **339**, 587.

Sternberg, M. J. and Gullick, W. J. (1990). A sequence motif in the transmembrane region of growth factor receptors with tyrosine kinase activity mediates dimerization. *Protein Eng* **3**, 245-8.

Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A. and Schwartz, A. L. (1996). The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. *Embo J* **15**, 3806-12.

Tanowitz, M. and Von Zastrow, M. (2002). Ubiquitination-independent trafficking of G protein-coupled receptors to lysosomes. *J Biol Chem* **277**, 50219-22.

Terrell, J., Shih, S., Dunn, R. and Hicke, L. (1998). A function for monoubiquitination in the internalization of a G protein-coupled receptor. *Mol Cell* **1**, 193-202.

Thien, C. B. and Langdon, W. Y. (2001). Cbl: many adaptations to regulate protein tyrosine kinases. *Nat Rev Mol Cell Biol* **2**, 294-307.

Thien, C. B., Walker, F. and Langdon, W. Y. (2001). RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation. *Mol Cell* **7**, 355-65.

Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R. C. et al. (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* **269**, 230-4.

Trub, T., Choi, W. E., Wolf, G., Ottinger, E., Chen, Y., Weiss, M. and Shoelson, S. E. (1995). Specificity of the PTB domain of Shc for beta turn-forming pentapeptide motifs amino-terminal to phosphotyrosine. *J Biol Chem* **270**, 18205-8.

Tsygankov, A. Y., Teckchandani, A. M., Feshchenko, E. A. and Swaminathan, G. (2001). Beyond the RING: CBL proteins as multivalent adapters. *Oncogene* **20**, 6382-402.

Tzahar, E., Pinkas-Kramarski, R., Moyer, J. D., Klapper, L. N., Alroy, I., Levkowitz, G., Shelly, M., Henis, S., Eisenstein, M., Ratzkin, B. J. et al. (1997). Bivalence of EGF-like ligands drives the ErbB signaling network. *Embo J* **16**, 4938-50.

Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J. and Yarden, Y. (1996). A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol* **16**, 5276-87.

Tzahar, E. and Yarden, Y. (1998). The ErbB-2/HER2 oncogenic receptor of adenocarcinomas: from orphanhood to multiple stromal ligands. *Biochim Biophys Acta* **1377**, M25-37.

Ullrich, A. and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-12.

van der Geer, P. and Pawson, T. (1995). The PTB domain: a new protein module implicated in signal transduction. *Trends Biochem Sci* **20**, 277-80.

van der Geer, P., Wiley, S., Gish, G. D., Lai, V. K., Stephens, R., White, M. F., Kaplan, D. and Pawson, T. (1996). Identification of residues that control specific binding of the Shc phosphotyrosine-binding domain to phosphotyrosine sites. *Proc Natl Acad Sci U S A* **93**, 963-8.

van der Geer, P., Wiley, S., Lai, V. K., Olivier, J. P., Gish, G. D., Stephens, R., Kaplan, D., Shoelson, S. and Pawson, T. (1995). A conserved amino-terminal Shc domain binds to phosphotyrosine motifs in activated receptors and phosphopeptides. *Curr Biol* **5**, 404-12.

Varmus, H. E., Bishop, J. M. and Vogt, P. K. (1973). Appearance of virus-specific DNA in mammalian cells following transformation by Rous sarcoma virus. *J Mol Biol* **74**, 613-26.

Venter, D. J., Tuzi, N. L., Kumar, S. and Gullick, W. J. (1987). Overexpression of the c-erbB-2 oncoprotein in human breast carcinomas: immunohistological assessment correlates with gene amplification. *Lancet* **2**, 69-72.

Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D. and Kuriyan, J. (1993). Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. *Cell* **72**, 779-90.

Waterman, H., Katz, M., Rubin, C., Shtiegman, K., Lavi, S., Elson, A., Jovin, T. and Yarden, Y. (2002). A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling. *Embo J* **21**, 303-13.

Waterman, H., Levkowitz, G., Alroy, I. and Yarden, Y. (1999). The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. *J Biol Chem* **274**, 22151-4.

Waterman, H. and Yarden, Y. (2001). Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases. *FEBS Lett* **490**, 142-52.

Webster, M. A., Cardiff, R. D. and Muller, W. J. (1995). Induction of mammary epithelial hyperplasias and mammary tumors in transgenic mice expressing a murine

mammary tumor virus/activated c-src fusion gene. *Proc Natl Acad Sci U S A* **92**, 7849-53.

Weiner, D. B., Kokai, Y., Wada, T., Cohen, J. A., Williams, W. V. and Greene, M. I. (1989a). Linkage of tyrosine kinase activity with transforming ability of the p185neu oncoprotein. *Oncogene* **4**, 1175-83.

Weiner, D. B., Liu, J., Cohen, J. A., Williams, W. V. and Greene, M. I. (1989b). A point mutation in the neu oncogene mimics ligand induction of receptor aggregation. *Nature* **339**, 230-1.

Weissman, A. M. (2001). Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* **2**, 169-78.

Wells, A., Welsh, J. B., Lazar, C. S., Wiley, H. S., Gill, G. N. and Rosenfeld, M. G. (1990). Ligand-induced transformation by a noninternalizing epidermal growth factor receptor. *Science* **247**, 962-4.

Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B. et al. (1992). Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell* **69**, 559-72.

Whistler, J. L., Enquist, J., Marley, A., Fong, J., Gladher, F., Tsuruda, P., Murray, S. R. and Von Zastrow, M. (2002). Modulation of postendocytic sorting of G protein-coupled receptors. *Science* **297**, 615-20.

Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol* **225**, 361-73.

Williams, L. T. (1989). Signal transduction by the platelet-derived growth factor receptor. *Science* **243**, 1564-70.

Woldeyesus, M. T., Britsch, S., Riethmacher, D., Xu, L., Sonnenberg-Riethmacher, E., Abou-Rebyeh, F., Harvey, R., Caroni, P. and Birchmeier, C. (1999). Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. *Genes Dev* **13**, 2538-48.

Woolford, J., McAuliffe, A. and Rohrschneider, L. R. (1988). Activation of the feline c-fms proto-oncogene: multiple alterations are required to generate a fully transformed phenotype. *Cell* **55**, 965-77.

Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T. and Toyoshima, K. (1986). Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature* **319**, 230-4.

Yarden, Y. and Schlessinger, J. (1987a). Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. *Biochemistry* **26**, 1443-51.

Yarden, Y. and Schlessinger, J. (1987b). Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. *Biochemistry* **26**, 1434-42.

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

Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W. C., Zhang, H., Yoshimura, A. and Baron, R. (1999). Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. *J Biol Chem* **274**, 31707-12.

Yokouchi, M., Kondo, T., Sanjay, A., Houghton, A., Yoshimura, A., Komiya, S., Zhang, H. and Baron, R. (2001). Src-catalyzed phosphorylation of c-Cbl leads to the interdependent ubiquitination of both proteins. *J Biol Chem* **276**, 35185-93.

Yoon, C. H., Lee, J., Jongeward, G. D. and Sternberg, P. W. (1995). Similarity of sli-1, a regulator of vulval development in C. elegans, to the mammalian proto-oncogene c-cbl. *Science* **269**, 1102-5.

Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W. and Schreiber, S. L. (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. *Cell* **76**, 933-45.

Zeillinger, R., Kury, F., Czerwenka, K., Kubista, E., Sliutz, G., Knogler, W., Huber, J., Zielinski, C., Reiner, G., Jakesz, R. et al. (1989). HER-2 amplification, steroid receptors and epidermal growth factor receptor in primary breast cancer. *Oncogene* **4**, 109-14.

Zhang, D., Sliwkowski, M. X., Mark, M., Frantz, G., Akita, R., Sun, Y., Hillan, K., Crowley, C., Brush, J. and Godowski, P. J. (1997). Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4. *Proc Natl Acad Sci U S A* **94**, 9562-7.

Zhang, H. T., O'Rourke, D. M., Zhao, H., Murali, R., Mikami, Y., Davis, J. G., Greene, M. I. and Qian, X. (1998). Absence of autophosphorylation site Y882 in the p185neu oncogene product correlates with a reduction of transforming potential. *Oncogene* **16**, 2835-42.

Zhang, K., Sun, J., Liu, N., Wen, D., Chang, D., Thomason, A. and Yoshinaga, S. K. (1996). Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. *J Biol Chem* **271**, 3884-90.

Zhao, Y. Y., Sawyer, D. R., Baliga, R. R., Opel, D. J., Han, X., Marchionni, M. A. and Kelly, R. A. (1998). Neuregulins promote survival and growth of cardiac myocytes. Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. *J Biol Chem* **273**, 10261-9.

Zheng, N., Wang, P., Jeffrey, P. D. and Pavletich, N. P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* **102**, 533-9.

Zhou, M. M., Ravichandran, K. S., Olejniczak, E. F., Petros, A. M., Meadows, R. P., Sattler, M., Harlan, J. E., Wade, W. S., Burakoff, S. J. and Fesik, S. W. (1995). Structure and ligand recognition of the phosphotyrosine binding domain of Shc. *Nature* **378**, 584-92.

Zrihan-Licht, S., Deng, B., Yarden, Y., McShan, G., Keydar, I. and Avraham, H. (1998). Csk homologous kinase, a novel signaling molecule, directly associates with the activated ErbB-2 receptor in breast cancer cells and inhibits their proliferation. *J Biol Chem* **273**, 4065-72.