# MECHANISMS INVOLVED IN ENDOCYTOSIS OF ERBB PROTEINS

by

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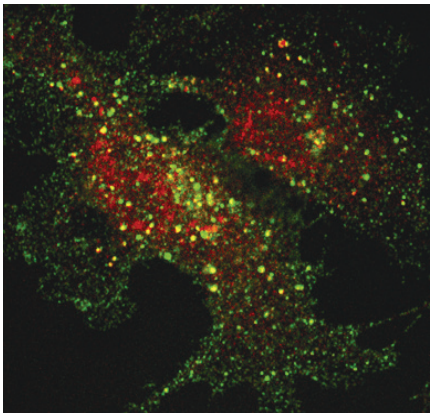

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Oslo, April 2008

Kamilla Breen

# TABLE OF CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| AP2 | Adaptor protein 2 |
| CHC | Clathrin heavy chain |
| CLC | Clathrin light chain |
| DAG | Diacylglycerol |
| EEA1 | Early endosome antigen 1 |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ESCRT | Endosomal sorting complex required for transport |
| GA | Geldanamycin |
| GPI | Glycosyl phosphatidylinositol |
| Hrs | Hepatocyte growth factor regulated tyrosine kinase substrate |
| IP3 | Inositol-(1,3,5)-trisphosphate |
| MAPK | Mitogen activated protein kinase |
| MEK | Mitogen activated protein kinase / Erk kinase |
| MHC-I | Major histocompatibility complex I |
| MVB | Multivesicular body |
| PAE | Porcine aortic endothelial |
| PI | Phosphatidylinositol |
| PIP | Phosphatidylinositol phosphate |
| PI(3)K | Phosphatidylinositol-3-kinase |
| PIP2 | Phosphatidylinositol-4,5-bisphosphate |
| PIP3 | Phosphatidylinositol-3,4,5-trisphosphate |
| PKC | Protein kinase C |
| PLC-γ | Phospholipase C γ |
| PTB | Phosphotyrosine binding |
| pY | Phosphotyrosine |
| SH2 | Src-homology 2 |
| siRNA | Silent inducing/ silent interfering RNA |
| STAM | Signal-transducing adaptor molecule |
| TGF-α | Transforming growth factor-α |
| TGN | Trans golgi network |
| TULA | T-cell ubiquitin ligand |
| UIM | Ubiquitin interacting motif |

# PUBLICATIONS INCLUDED

**Paper I** Camilla Haslekås[1], **Kamilla Breen**[1], Ketil W. Pedersen, Lene E. Johannessen, Espen Stang, and Inger Helene Madshus.
[1] These authors contributed equally to this study

The inhibitory effect of ErbB2 on epidermal growth factor-induced formation of clathrin-coated pits correlates with retention of epidermal growth factor receptor-ErbB2 oligomeric complexes at the plasma membrane.

*Molecular Biology of the Cell,* Vol. 16, 5832–5842, December 2005.

**Paper II** **Kamilla Breen**, Sissel B Rønning, Nina Marie Pedersen, Espen Stang and Inger Helene Madshus.

ErbB3 is constitutively endocytosed in a clathrin-dependent manner.

*Manuscript*

**Paper III** Nina Marie Pedersen[1], **Kamilla Breen**[1], Camilla Haslekås, Espen Stang and Inger Helene Madshus.
[1] These authors contributed equally to this study.

Expression of EGFR or ErbB3 facilitates Geldanamycin-induced downregulation of ErbB2.

*Submitted*

**Paper IV** Vibeke Bertelsen, **Kamilla Breen**, Kirsten Sandvig, Espen Stang and Inger Helene Madshus.

The Cbl-interacting protein TULA inhibits dynamin-dependent endocytosis.

*Experimental cell research,* Vol. 313, 1696-1709, February 2007

## SUMMARY

Cell growth, differentiation and signaling are important processes needed for cell survival. These processes are mediated by binding of growth factors to receptors on the cell membrane. One group of such receptors is the ErbB proteins, which are involved in a wide array of different cellular functions. There are four different ErbB proteins, EGFR, ErbB2, ErbB3 and ErbB4. The ErbB proteins dimerize with and activate each other, leading to activation of intracellular signaling pathways. Downregulation of the receptors from the plasma membrane and a subsequent degradation is an important way to attenuate the signaling. EGFR is known to be downregulated by endocytosis through clathrin-coated pits, and ubiquitination has been suggested to be important for endocytosis. For the other receptors the mechanism of endocytosis is not clear. ErbB proteins have been shown to be related to a range of different cancer types. Dysregulation of ErbB proteins can lead to aberrant signaling and increased cell growth and survival. Knowledge about their regulation is therefore important to be able to target these cancers with specific drugs.

All papers in this work concern endocytosis of the ErbB proteins. ErbB2 is endocytosis resistant, and this ability also affects EGFR in heterodimers with ErbB2. We studied these dimers and found that a reason why ErbB2 inhibits endocytosis of EGFR is that it retains EGFR at the plasma membrane by prohibiting EGF-induced formation of clathrin-coated pits (Paper I). We continued by investigating whether this might also be the case for ErbB3, which is also reported to be endocytosis impaired but we did not find the same correlation. In contrast, we found that endocytosis of ErbB3 was a constitutive process dependent on clathrin (Paper II). We also investigated the endocytosis of ErbB2 itself by inducing its endocytosis with the benzoquinone ansamycin Geldanamycin (GA). Also in this case we found a clear correlation between endocytosis and the nature of the heterodimerization partner. In contrast to cell expressing only ErbB2, cells co-expressing EGFR and/or ErbB3 showed a significant increase in the rate of GA-induced endocytosis of ErbB2 (Paper III). Finally, we investigated the importance of ubiquitin for endocytosis of EGFR and our results support that ubiquitination of EGFR is important for its endocytosis (Paper IV).

# 1 INTRODUCTION

All living organisms are products of repeated rounds of cell divisions. The processes of growth, cell division and differentiation are crucial in early steps of development, but also in adult living organisms cell division is a prerequisite for life. There is a continuous need for growth and renewal of cells and every second several millions of new cells are produced. If all cell divisions stopped, we would die within a few days (Alberts 2002). The signals for cell growth and division are mediated by growth factors. A growth factor is a naturally occurring protein capable of stimulating cell proliferation and cell differentiation. They are produced by the cells in the organism itself or supplied by food. These growth factors, or growth factor receptor ligands, bind more or less specific growth factor receptors located on the cell membrane, and the growth factor receptors then transmit a signal to the interior of the cell. This is a way of transmitting signals between cells, or in the cell itself by binding its own produced ligands. This process must be under tight control not only to sustain the need for cell renewal, but also to stop cell division when not needed any longer. Excessive cell divisions can lead to oncogenesis.

One major group of growth factor receptors is the ErbB protein group, named so because of their homology to the erythroblastoma viral protein, v-erbB. The ErbB protein group comprises four members with close sequence homology: Epidermal growth factor receptor (EGFR, also termed ErbB1 or HER1) ErbB2 (also termed HER2 or Neu), ErbB3 (or HER3) and ErbB4 (or HER4). There are 14 different ligands able to bind one or more of the four ErbB proteins. Aberrant regulation of all four of these growth factor receptors has been correlated with human cancers (Hynes et al. 2005).

There are several ways in which aberrant regulation of ErbB proteins might be involved in cancer. First, the cells may overexpress the receptor, leading to increased signaling. This can be a result of gene amplification and/or altered transcription and translation. Second, deletions or mutations in the gene encoding the receptor may occur. The result can be a receptor that is either constitutively active and/or a receptor that escapes downregulation, again leading to increased signaling. Co-overexpression of a receptor

and its ligand is a third way that can lead to increased signaling. The final output of all these possible ways of having increased or changed signaling may give the cells new properties like increased growth, prolonged survival and/or enhanced migration (reviewed in Zandi et al. 2007). Attenuation of ErbB signaling is therefore a key to avoid cancer. Downregulation by endocytosis and subsequent degradation of the receptors is a major way of mediating attenuation. All the ErbB proteins except for EGFR are reported to be endocytosis-impaired and their regulation is therefore an important issue.

## 1.1 THE ERBB FAMILY MEMBERS

The ErbB proteins and their ligands are expressed in almost all kinds of tissues and serve important physiological functions. They were first found to be important in neurons and are still also referred to as neu-receptors. In addition to their importance in neuronal migration in the brain and in the synapses, they are also important in the mammary gland and for proper development of the heart of embryos (reviewed in Burden et al. 1997). The ErbB proteins comprise four extracellular domains (Figure 1 A). L1 and L2 bind to ligand and the cysteine rich regions CR1 and CR2 mediate dimerization. An intracellular kinase domain with tyrosine kinase activity is localized in the N-terminal part of the intracellular domain. The ErbB proteins can obtain two alternative conformations, the closed or autoinhibited conformation (accessible for ligand binding and with CR-domains embedded in the molecule) and the open conformation (not accessible for further ligand binding and with the dimerization arm exposed) (Ogiso et al. 2002). Binding of ligand stabilizes the closed conformation and thereby dimerization of receptors, promoting tyrosine phosphorylation by the tyrosine kinase domains (Figure 1B). Receptor phosphorylation is the primary step initiating the cascade of intracellular signaling.

EGFR it the most extensively studied of the four ErbB proteins and much is known about its signaling and attenuation. However, despite the similar structures, not all information is applicable to the other ErbB proteins. This introduction will first contain a short part on each ErbB protein before signaling and downregulation will be discussed

in more detail for EGFR, with discrepancies to the other receptors specified where knowledge is available.

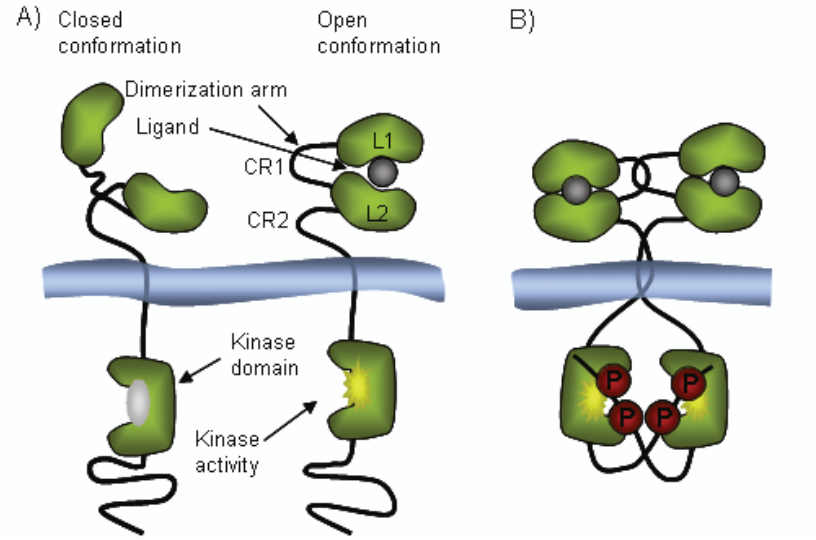


**Figure 1. The general structure of ErbB proteins. A**. The receptors comprise two ligand binding domains (L1 and L2) that mediate interaction with ligand and two cyctein rich domains (CR1 and CR2) that mediate interaction with another receptor. In the intracellular region, the receptor comprises a kinase domain with kinase activity. **B**. Upon ligand binding and dimerization the C-terminal tails are phosphorylated by the intrinsic or neighbouring kinase domain.

### 1.1.1 EGFR

**Structural properties of EGFR**

Of the four ErbB proteins, EGFR was the first receptor to be discovered. Epidermal growth factor (EGF), was discovered in the early sixties (Cohen 1962; Cohen 1964) and some years later ErbB1 was discovered to be its receptor (Carpenter et al. 1978). Now the receptor is most often referred to as EGFR. EGFR is a highly glycosylated 170 kDa protein which in addition to EGF has six other ligands (Figure 2), all of them affecting EGFR in a distinct way (reviewed in Yarden 2001a). When bound to a ligand, the dimerization arm is exposed. This mediates dimerization, either homodimerization or heterodimerization with one of the other ErbB proteins.

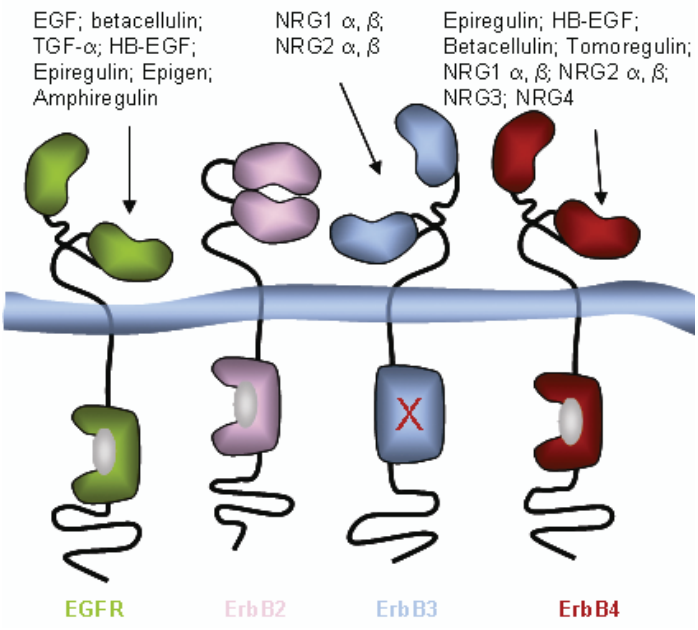


**Figure 2. The ErbB protein family.** The ErbB proteins have distinct propertied. ErbB1, ErbB3 and ErbB4 bind a distinct set of ligands, whereas ErbB2 does not bind any ligand. The receptors contain an intracellular kinase domain, but the kinase activity of ErbB3 is impaired.

**Regulation and trafficking of EGFR**

Upon binding of a ligand to EGFR, the receptor is activated and taken into the cell mainly by clathrin-mediated endocytosis. The nature of the ligand can determine the fate of the receptor -either recycling or degradation. The attachment of ubiquitin molecules to the receptor is necessary for degradation and is mediated by the ubiquitin ligase Cbl, which is recruited to the EGFR upon activation. (Levkowitz et al. 1996; Waterman et al. 2002). The binding between EGF and EGFR is stable in endosomes and the EGF-bound form of EGFR remains ubiquitinated on endosomes, translocated to inner vesicles of multivesicular bodies (MVBs) and eventually degraded in lysosomes (reviewed in Sorkin et al. 2002). Stimulation with EGF thus reduces the half life of the receptor from ~10 hours (Citri et al. 2002) to 1.5 – 2 hours (Huang et al. 2006). In contrast, when EGFR is endocytosed upon binding transforming growth factor-α (TGF-α), the acidic pH in endosomes mediates detachment of TGF-α from the receptor (Ebner

et al. 1991; French et al. 1995), which in turn leads to deubiquitination (Alwan et al. 2003) and therefore reduced ability for EGFR to bind to the endosomal sorting machinery. The EGFR is then recycled back to the plasma membrane (section 1.6.2).

**EGFR and cancer**

The EGFR is involved in various cancer forms (Prenzel et al. 2001; Zandi et al. 2007) resulting from both overexpression and mutations of the receptor. Overexpression of the EGFR has been found to correlate with decreased survival in head and neck, bladder, cervical, oesophageal and ovarian cancers (Nicholson et al. 2001). Several variants of mutated EGFR have been detected in various cancers. One of the most studied mutated forms of EGFR is the EGFRvIII. In this variant of EGFR, the exons encoding the L1 and ⅔ of the CR1 domain are deleted (Wong et al. 1992). The receptor is constitutively phosphorylated and escapes downregulation due to impaired endocytosis and increased recycling (Grandal et al. 2007) caused by inefficient ubiquitination (Han et al. 2006; Grandal et al. 2007). A range of other changes have been found within the EGFR gene, including point mutations, deletions and duplications (reviewed in Zandi et al. 2007). Many of the EGFR variants containing deletions lack all or parts of the extracellular domain which impairs the ligand-induced downregulation (Wong et al. 1992; Frederick et al. 2000). Antibodies that bind to EGFR and inhibit its ligand binding, and thus signaling, have been used for treatment of cells overexpressing EGFR (Goldstein et al. 1995; Narita et al. 2001). One such antibody, which has been approved for clinical use, is Cetuximab. The antibody binds to the extracellular L2 domain (Li et al. 2005) and has anti-tumor effects in combination with chemotherapy or radiotherapy (Baselga 2001; Prewett et al. 2002). To target the kinase activity of the EGFR in cancer cells, tyrosine kinase inhibitors that disrupt the activation of EGFR are used. Two examples that are approved for clinical use are Gefitinib and Erlotinib (reviewed in Arora et al. 2005).

### 1.1.2 ERBB2

**Structural properties of ErbB2**

Because of the tethered structure of the 185 kDa ErbB2, this receptor can not bind ligand (Klapper et al. 1999; Garrett et al. 2003). In spite of this apparent deficiency, the special structure of ErbB2 makes it particularly frequent in severe cancer forms. First, it has its dimerization arm constitutively exposed (Figure 2) similar to the ligand bound form of EGFR and ErbB3 (Schlessinger 2002; Garrett et al. 2003). This property makes ErbB2 the preferred heterodimerization partner in the receptor family (Sliwkowski et al. 1994; Yarden 2001b; Yarden et al. 2001). Second, it seems that a wider range of phospho-tyrosine binding proteins are recruited to this receptor compared to the other receptors in the family (Jones et al. 2006). Third, when overexpressed, ErbB2 is reported to increase binding affinity of heterodimerization partners and thus sustain signaling from heterodimers (Karunagaran et al. 1996) and also to lower the ligand specificity of the dimerization partner (Alimandi et al. 1997; Pinkas-Kramarski et al. 1998; Wang et al. 1998). Fourth, when overexpressed, ErbB2 is found to be constitutively activated (Yuste et al. 2005). Finally, ErbB2 escapes downregulation (Sorkin et al. 1993; Baulida et al. 1996; Austin et al. 2004; Hommelgaard et al. 2004; Longva et al. 2005). The ability also affects EGFR in heterodimers with ErbB2 (Muthuswamy et al. 1999; Wang et al. 1999; Worthylake et al. 1999), giving these heterodimers prolonged time to signal. ErbB2 has been claimed not to be able to homodimerize (Burgess et al. 2003), but was recently found in homodimers as a result of high overexpression in breast cancer cells (Yang et al. 2007).

**Regulation and trafficking of ErbB2**

In contrast to EGFR for which Cbl is the main ubiquitin ligase, the chaperone interacting protein (CHIP) is involved in ubiquitination of ErbB2 (Xu et al. 2002; Zhou et al. 2003). Even though the half-life of ErbB2 is reported as shorter than that of non-stimulated EGFR, between 4 hours (Citri et al. 2002) and 7 hours (Baulida et al. 1996; Nielsen et al. 2003), it still inhibits endocytosis of the EGFR. The reason why ErbB2 is endocytosis resistant and what makes it interfere with the endocytosis of activated EGFR is currently not understood. There are indications that it may be a result of the localization to areas outside clathrin-coated pits (Hommelgaard et al. 2004; Offterdinger

et al. 2007) or that there are some signals for endocytosis missing in the C-terminal of ErbB2 (Sorkin et al. 1993). The stability of ErbB2 is constantly dependent on interaction with heat shock protein 90 (Hsp90) (Citri et al. 2002). GA binds to and inhibits the stabilizing action of Hsp90 (Xu et al. 2001) and is shown to reduce ErbB2 half-life from 4 hours to approximately 1.5 hours (Citri et al. 2002). It is shown that Hsp90 cycles between two conformations, an ATP and an ADP bound form (reviewed in Powers et al. 2007). When in the ADP-bound form Hsp90 also associates with among others Hsp70 and CHIP and this form mediates destabilization of client proteins by ubiquitination which target the proteins for proteasomal degradation (reviewed in Murata et al. 2001). GA stabilizes the ADP and Hsp70 bound form of Hsp90 (Figure 3) (reviewed in Isaacs et al. 2003). Upon GA-treatment, ErbB2 is downregulated by mechanisms not fully understood. It is shown that ErbB2 is ubiquitinated by CHIP upon GA-treatment (Mimnaugh et al. 1996) and also that GA induces recruitment of CHIP to ErbB2 (Xu et al. 2002). Several other steps have also been reported, including endocytosis (Longva et al. 2005) and either lysosomal or proteazomal degradation (Mimnaugh et al. 1996; Lerdrup et al. 2006). The detailed mechanism of GA-induced downregulation of ErbB2 is not known and has been addressed in Paper III.

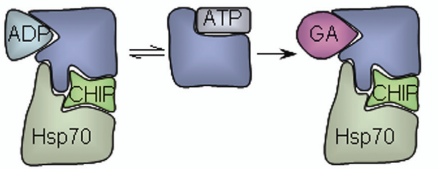


**Figure 3. Simplified model of Hsp90 cycling.** Hsp90 (blue) cycles between two conformations, the ATP bound and the ADP/Hsp70/CHIP-bound. GA stabilizes the Hsp70/CHIP bound form.

**ErbB2 and cancer**

ErbB2 is overexpressed in 20-30 % of breast and ovarian tumors (Witton et al. 2003; Abd El-Rehim et al. 2004), but is also found overexpressed in bladder, colon, pancreas, gastric and endometrial tumors (Junttila et al. 2003; Holbro et al. 2004; Roskoski 2004). In these cancers, ErbB2 has been linked to therapeutic resistance and poor prognosis (Ross et al. 2003). For this reason, ErbB2 has been considered an important therapeutic target for several years. Several drugs and antibodies are used clinically to target ErbB2. Two examples are Trastuzumab (or Herceptin) which inhibits signaling from ErbB2 and

Pertuzumab (or Omnitarg) that inhibits dimerization of ErbB2 and thereby its signaling (reviewed in Hynes et al. 2005). Also derivates of GA seem promising in treatment of cancer cells with overexpression of ErbB2 (Neckers 2002).

### 1.1.3 ERBB3

**Structural properties of ErbB3**

ErbB3 (with a molecular weight of 180 kDa) differs from EGFR, ErbB2 and ErbB4 in that it contains a mutation in the kinase domain making the receptor kinase dead or kinase defective (Guy et al. 1994; Sliwkowski et al. 1994). ErbB3 can interact with two of the neuregulin ligands (also called heregulins), NRG1 and NRG2 (Figure 2). ErbB3 is reported not to homodimerize in response to heregulin binding (Berger et al. 2004). For this reason, and since the kinase activity is impaired, it depends on a heterodimerization partner in order to be activated (Kim et al. 1998).

**Regulation and trafficking of ErbB3**

As ErbB2, also ErbB3 is considered to be endocytosis impaired (Baulida et al. 1996; Waterman et al. 1998) as internalization of heregulin is slow compared to internalization of EGF (Baulida et al. 1997). Whether the slow rate of endocytosis also affects EGFR in EGFR/ErbB3 dimers is not known and was part of the investigation in Paper II. There is evidence that heregulin, and thus possibly ErbB3, is more effectively recycled after internalization than both EGF and TGF-α (Waterman et al. 1998). ErbB3 does not contain binding sites for Cbl, but instead binds another ubiquitin ligase called Nrdp1. Nrdp1 promotes degradation of ErbB3, possibly proteasome-dependently (Diamonti et al. 2002; Qiu et al. 2002). The specific binding site of Nrdp1 has not been determined, but is localized to a region between the kinase domain and the transmembrane domain (Bouyain et al. 2007). The expression level of Nrdp1 has been shown to be positively effected by heregulin, thereby providing a sort of 'ligand induced' ubiquitination and downregulation of ErbB3 (Cao et al. 2007). However, there are also reports concluding that heregulin does not significantly affect downregulation of ErbB3 (Baulida et al. 1997; Qiu et al. 2002). The reason for this discrepancy may be differences in Nrdp1 expression. Tumors overexpressing ErbB3 was in 70 % of the cases correlated with suppressed level of Nrdp1 (Yen et al. 2006), rendering Nrdp1-

mediated ErbB3 degradation dysfunctional. Heregulin is also reported to dissolve constitutive oligomers of ErbB3 (Landgraf et al. 2000; Kani et al. 2005), although a more recent report argues that heregulin makes ErbB3 cluster at the plasma membrane (Yang et al. 2007). There are thus a lot of unanswered questions concerning endocytosis and regulation of ErbB3. Endocytosis of ErbB3 is investigated in Paper II.

**ErbB3 and cancer**

ErbB3 has been found to be overexpressed in multiple forms of cancers like colon, bladder and prostate cancers and it is reported that ErbB3 overexpression occurs in 63 % of breast tumors (Yen et al. 2006). There are still no known mutations or gene amplification detected for ErbB3 (reviewed in Sweeney et al. 2006). Recent studies also indicate that overexpression of ErbB3 correlates with metastases and reduced survival (Witton et al. 2003; Wiseman et al. 2005). ErbB3/ErbB2 dimers are particularly potent in mitogenic signaling (Wallasch et al. 1995; Pinkas-Kramarski et al. 1996) and many ErbB2 positive tumors are also positive for ErbB3 (Naidu et al. 1998; deFazio et al. 2000; Bieche et al. 2003; Holbro et al. 2003), giving these two receptors a special relation. There are several reasons for their potency (reviewed in Citri et al. 2003). First, they activate signaling pathways necessary for proliferation and survival. Second, they display no or low ligand-induced downregulation (Baulida et al. 1997). Third, they have a slow rate of ligand dissociation (Sliwkowski et al. 1994; Tzahar et al. 1996) and fourth, the presence of ErbB2 widens the spectrum of ligands that can bind to ErbB3 (Alimandi et al. 1997; Pinkas-Kramarski et al. 1998; Wang et al. 1998).

### 1.1.4 ERBB4

**General overview**

ErbB4 is the least studied of the four ErbB proteins. It binds a wide range of different ligands (Figure 2) and it has an active kinase domain. Although there is only one gene encoding ErbB4 (Zimonjic et al. 1995), several ErbB4 isoform have been described and are probably a result of alternative RNA splicing (Elenius et al. 1997; Elenius et al. 1999; Rio et al. 2000). The ligand-induced endocytosis of ErbB4 is slow, and the half-life of a chimeric receptor of EGFR/ErbB4 (EGFR extracellular domain and ErbB4 intracellular domain) was determined to 6 hours in non-stimulated cells and 5.5 hours in

EGF-treated cells (Baulida et al. 1996) ErbB4 is unable to recruit Cbl (Levkowitz et al. 1996), but it might, as ErbB3, be regulated by Nrdp1, since overexpression of Nrdp1 was reported to reduce the level of ErbB4 (Diamonti et al. 2002). ErbB4 is upon ligand binding, proteolytically cleaved, generating an 80 kDa protein (s80) that translocates to the nucleus where it is frequently observed in cancer cells. In the nucleus, s80 can promote transcription by functioning as a chaperone that facilitates nuclear entry of different transcription factors (Ni et al. 2001; Omerovic et al. 2004; Williams et al. 2004; Aqeilan et al. 2005). The role of ErbB4 in human cancer is debated. Overexpression has been reported in childhood medulloblastoma where it correlates with aggressive tumor type and metastases (Gilbertson et al. 1997; Gilbertson et al. 2001). However, ErbB4 has also been reported to inhibit proliferation and was recently suggested do be a tumor suppressor (Suo et al. 2002; Williams et al. 2003; Barnes et al. 2005). A more recent study does, however, correlate expression of ErbB4 with severe, metastatic and vascular invasive cancers in a high number of breast carcinomas (Abd El-Rehim et al. 2004).

Although all ErbB proteins have different functions and can give distinct outcome upon overexpression, it has been difficult to assign a certain property to one receptor in particular, as they are often overexpressed together. Furthermore, their interplay is significant. It is therefore recommended that an expression profile of all ErbB members should be determined to decide on treatment of individual cancers (Witton et al. 2003; Zaczek et al. 2005).

## 1.2 LIGAND BINDING AND RECEPTOR DIMERIZATION

In the absence of ligand, the ErbB proteins are in general thought to be monomeric, and the receptors adopt the tethered or closed conformation, inhibiting dimerization (Cho et al. 2002; Ferguson et al. 2003; Bouyain et al. 2005). The binding of ligand to EGFR induces a conformational change and stabilizes the open conformation of the receptor, easing dimerization and oligomerization, and thus phosphorylation (Schlessinger 2002) (reviewed in Burgess et al. 2003; Dawson et al. 2007). ErbB2 is an exception since its constitutively open structure prohibits ligand binding, and thus ligand-induced

dimerization is induced through its dimerization partner (reviewed in Yarden et al. 2001). The receptors have traditionally been considered unable to dimerize in the absence of ligand, but there is evidence that a small fraction of the receptors exist in a open conformation also in the absent of ligand, making dimerization possible also in unstimulated cells, and especially in cells overexpressing the receptors (Yu et al. 2002; Klein et al. 2004). There are arguments, however, that simply adopting the open conformation is not sufficient for dimerization (Dawson et al. 2007).

## 1.3 THE PLASMA MEMBRANE

The papers included in this thesis are all focused on endocytosis of the ErbB proteins. To understand mechanisms of endocytosis, knowledge about the molecular composition of the plasma membrane is important. The plasma membrane consists of a double layer of lipids (including saturated and non-saturated fatty acids, cholesterol, sphingolipids and phosphoinositides (PIs)) and proteins, and the two layers are held together by non-covalent interactions (Figure 4). The lipids and proteins are organized into distinct areas and form regions of specific function (reviewed in Brown et al. 1998).

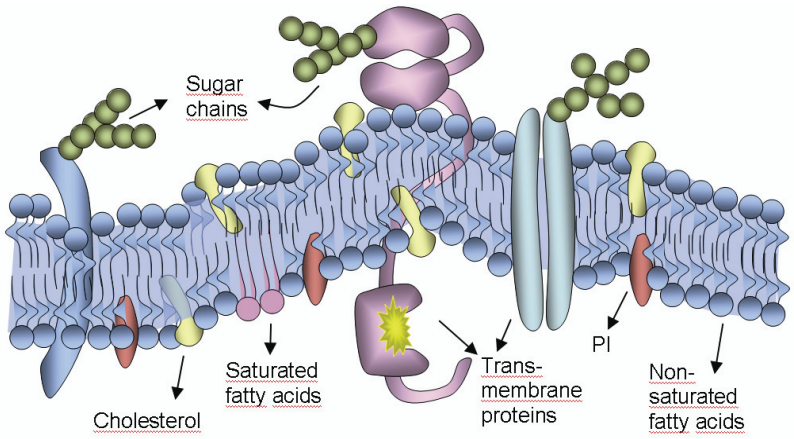


**Figure 4. The lipid bilayer constituting the plasma membrane.** The plasma membrane consists of an outer and inner layer of saturated and non-saturated fatty acids. The lipid polar heads face towards the cell surface or the cell interior and the nonpolar fatty acid chains face towards the other lipid layer. Proteins are also inserted in the membrane, along with accessory lipids like phosphoinositides (PIs) and cholesterol. Extracelluarly, most proteins contain sugar-chains.

### 1.3.1 PHOSPHOINISITIDES

The PIs of the cellular membranes come in different forms. Phosphorylation of one to three hydroxyl groups of the inositol-ring (position 3, 4 or 5), give rise to up to seven possible PIs (reviewed in Di Paolo et al. 2006). Several different protein domains, like FYVE, PH, PX, ENTH and ANTH domains, bind specific PIs (reviewed in Lemmon 2003). The distribution pattern of the different PIs to specific areas within the cell may therefore determine the localization of a protein (see Simonsen et al. 2001 for a review of the function of the PIs in each membrane compartment). $PI(4,5)P_2$ (or PIP2) is the PI most abundant at the plasma membrane and is necessary to recruit proteins important for endocytosis, like adaptor protein 2 (AP2) and epsin (Zoncu et al. 2007). PI(3)P is abundant in the early endosomes and targets early endosome antigen-1 (EEA1) to this compartment (Stenmark et al. 1996). PI(3)P is also important for endosomal sorting of proteins (Raiborg et al. 2001). Specific PI kinases (like PI(3)kinase) or phosphatases (like PTEN) can convert a PI from one form to another, thus making a new docking site for a PI-interacting protein. Membrane-localized proteins, including ErbB proteins, can be indirectly linked to a specific membrane compartment through interaction with a PI-interacting protein, like AP2 at the plasma membrane or AP1, which is localized to endosomes and the trans golgi network (TGN) (see Robinson 2004 for a review on adaptor proteins).

### 1.3.2 LOCALIZATION OF ERBB PROTEINS AT THE PLASMA MEMBRANE

The plasma membrane contains 'floating' areas rich in sphingolipids and cholesterol named rafts. In rafts, the fatty acids have a more ordered and extended conformation, possibly because of a higher concentration of saturated fatty acids (Lichtenberg et al. 2005). The association of caveolin with specific lipid rafts gives them a characteristic curved morphology and thereby forms a caveola (Kirkham et al. 2005). The caveolae invaginate and forms flask shaped structures, but their degree of internalization has been debated (see section 1.5.1) It has been reported that EGFR is localized to caveolae in unstimulated cells (Mineo et al. 1999; Smart et al. 1999) and that upon ligand binding, EGFR migrates out of caveolae (Mineo et al. 1999) and into the clathrin-coated pits (see section 1.5.1). The translocation of EGFR from caveolae was reported to depend on an

active receptor kinase domain and required activated protein kinase C (PKC) (Mineo et al. 1999). Others have, however, concluded that EGFR is not concentrated in caveolae and the results were obtained both by fractionation studies (Waugh et al. 1999) and by immuno electron microscopy studies (Ringerike et al. 2002). For the other ErbB proteins, little is known about the localization at the plasma membrane. However, it has been suggested that ErbB2 is localized to caveolae but fails to migrate out of these structures upon EGF stimulation (Mineo et al. 1999) and that it is raft-associated at membrane protrusions (Hommelgaard et al. 2004). A contradicting report says that ErbB2 and ErbB4 upon heregulin stimulation migrates from the bulk membrane and into caveolae or rafts (Zhou et al. 2001).

## 1.4 SIGNALING THROUGH ERBB PROTEINS

When dimers are formed, the intrinsic tyrosine kinase of the receptors is activated, promoting phosphorylation of the receptor tails (Sherrill et al. 1999). In each receptor, distinct tyrosines are phosphorylated and serve as docking sites for cytosolic signaling proteins containing Src-homology 2 (SH2) domains or phosphotyrosine-binding (PTB) domains (Figure 5). The recruited proteins include kinases that can phosphorylate serine and threonine residues and further increase the amount of docking sites. The pattern of phosphorylation is determined by the nature of the ligand and the dimerization partner and decides the outcome of the signaling process (Olayioye et al. 2000). Two major signaling pathways activated by the ErbB proteins are the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway. Depending on different phosphorylation patterns, the signaling mediates different cellular outcomes such as apoptosis, cell survival, migration, growth, adhesion or differentiation (reviewed in Yarden et al. 2001; Zahnow 2006).

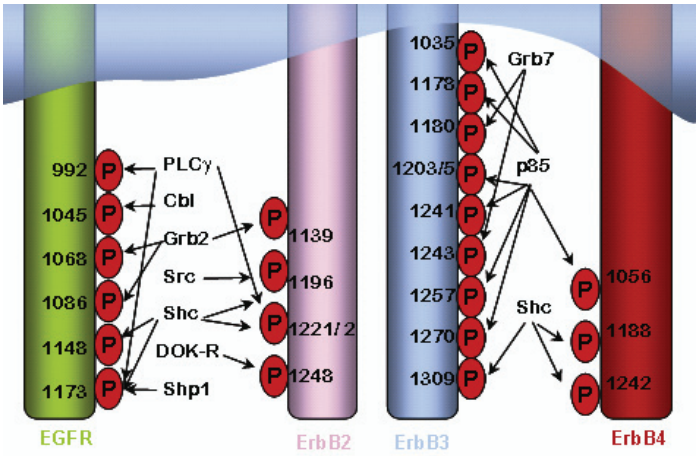


**Figure 5. Ligand-induced phosphorylation sites in the C-terminal tails of the ErbB proteins.** When receptors are activated, they are phosphorylated at the indicated tyrosine residues. The phosphotyrosines recruit different phosphotyrosine binding proteins involved in transmitting signaling or mediating receptor downregulation. The figure is based on (Olayioye et al. 2000) and (Hynes et al. 2005)

### 1.4.1 THE MAPK PATHWAY

Following activation of the EGFR, the SH2 domain of growth factor receptor-bound protein 2 (Grb2) binds directly to EGFR phosphotyrosine (pY) 1068 and/or 1086 (Batzer et al. 1994) or indirectly through phosphorylated Shc (Figure 5) (Sasaoka et al. 1994). Shc binds via its PTB domain to EGFR pY 1148 mediating activation of Shc (Figure 6). The binding of Grb2 and Shc recruits the nucleotide exchange factor SOS which mediates nucleotide exchange of Ras-GDP to Ras-GTP and thus Ras activation (reviewed in Nimnual et al. 2002). Activated Ras further activates the serine/threonine kinase Raf-1 (Hallberg et al. 1994) which activates MAP/Erk kinase (MEK). MEK eventually activates the MAPKs Erk1 and Erk2. Erk1 and Erk2 can enter the nucleus and activate transcription factors of specific genes (reviewed in Kolch 2000).

### 1.4.2 THE PLCγ AND PI3K PATHWAYS

Phospholipase C γ (PLCγ) is an enzyme which upon activation catalyzes hydroslysis of PIP2 to form 1,2-diacylglycerol (DAG) and inositol-(1,3,5)-trisphosphate (IP3). PLCγ binds directly to phosphotyrosines in EGFR and ErbB2 (Figure 5) and the receptors

promote PLCγ activation (Figure 6). IP3 mediates calcium release from intracellular stores, affecting a range of calcium-dependent enzymes, while DAG promotes activation of the serine/threonine kinase protein kinase C (PKC). PI3K is another kinase involved in the signaling from ErbB proteins. The p85 subunit of PI3K can be recruited to Cbl upon EGFR activation (Hartley et al. 1995; Fang et al. 2001), but the main way of activation is through the six binding sites for p85 in ErbB3 (Figure 5) (Ram et al. 2000). Upon binding of p85 to ErbB3, p85 is brought close to the active kinase domain of an ErbB3 heterodimerization partner and is activated by this dimerization partner. PI3K catalyses the phosphorylation of the 3' position of the inositol-ring of PIs and can generate $PI(3,4,5)P_3$ (PIP3). PIP3 is a binding site for the serine-threonine kinase Akt, thus recruiting Akt to the plasma membrane and thereby promoting its activation (Figure 2Figure 6). Akt is involved in regulation of several proteins affecting cell survival and proliferation (reviewed in Vivanco et al. 2002).

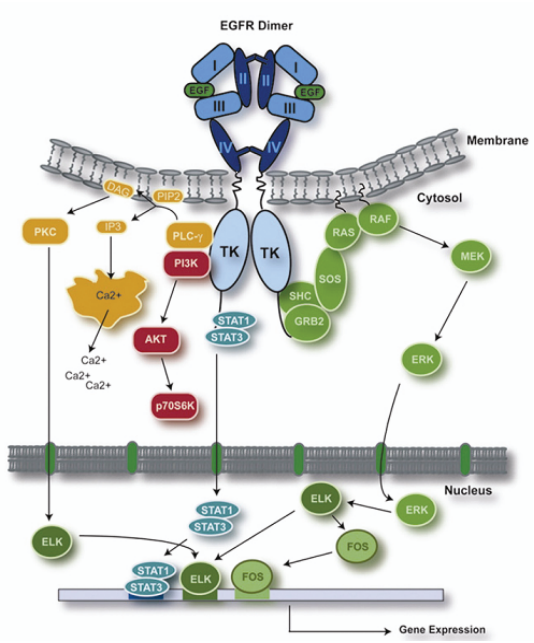


**Figure 6. Signaling path-ways activated by EGFR.** Three main signaling pathways of ErbB proteins are the MAPK pathway activating the MAPK Erk, the PI3K-Akt-pathway activating Akt and the STAT pathway. Activation of PLCγ is also involved in important steps mediating signaling. The signaling pathways can lead to phosphorylation of proteins entering the nucleus and affecting gene transcription. The figure is from (Zandi et al. 2007) and the figure legend is modified.

### 1.4.3 THE JAK AND STAT PAHWAYS

The STAT proteins are inactive transcription factors which are constitutively associated with EGFR (Figure 6). The STAT proteins may be activated upon activation of EGFR

(Olayioye et al. 1999; Xia et al. 2002) and JAK (reviewed in Leonard 2001) or possibly by Src (Figure 6) (Olayioye et al. 1999; Kloth et al. 2003). Upon activation, the STAT proteins translocate to the nucleus as homo- or heterodimers (Jorissen et al. 2003).

### 1.4.4 SRC-MEDIATED PHOSPHORYLATION

Src and other cytosolic tyrosine kinases are also involved in the signal transduction from the ErbB proteins. Src does not bind directly to the ligand-mediated phosphorylation sites of EGFR, but there is evidence that it phosphorylates tyrosine residues of the EGFR (such as Y890, Y920, Y845 and Y1101) thereby producing docking sites for the p85 subunit of PI3K as well as docking sites for Src itself (Lombardo et al. 1995; Stover et al. 1995; Biscardi et al. 1999). Src also phosphorylates and activates PI3K directly and thus activates the PI3K-pathway (Shoelson et al. 1993). Phosphorylation by Src seems to be particularly important for ErbB2. A recent paper indicates that the Src-mediated phosphorylation of Y877 mediates a conformational change in ErbB2 by twisting a loop of the receptor closer to the ErbB2 kinase domain, promoting further phosphorylation of ErbB2 (Xu et al. 2007).

## 1.5 ENDOCYTOSIS OF ERBB PROTEINS

To attenuate the signaling mediated by the activated ErbB proteins, the proteins can be downregulated from the plasma membrane by endocytosis. Endocytosis is a process where the cells absorb material into intracellular vesicles by engulfing the material with the plasma membrane. The vesicles formed, fuse and make larger endosomes where the cargo is sorted either for recycling back to the plasma membrane or for degradation in lysosomes. Endocytosis is divided into two main forms, phagocytosis and pinocytosis (Figure 7). Phagocytosis is often restricted to certain cell types, like phagocytes, and involves uptake of large particles like bacteria. Pinocytosis is the uptake of fluids and solutes and occurs in all cell types. Pinocytosis can be divided in four main mechanisms. Macropinocytosis is achieved by membrane ruffling and is often induced in cells upon stimulation, for instance by growth factors. The three remaining forms of pinocytosis all involve inward budding of the plasma membrane and includes caveolin-mediated endocytosis, clathrin mediated endocytosis (CME) and clathrin- and caveolin

independent endocytosis (reviewed in Conner et al. 2003a). The best characterized form of endocytosis is CME. This is the main way for EGFR (Carpentier et al. 1982; Hanover et al. 1984; Kazazic et al. 2006) and GA-treated ErbB2 to enter the cell (Pedersen et al. In press). The way ErbB3 is endocytosed has so far been unclear and has been studied in Paper II.

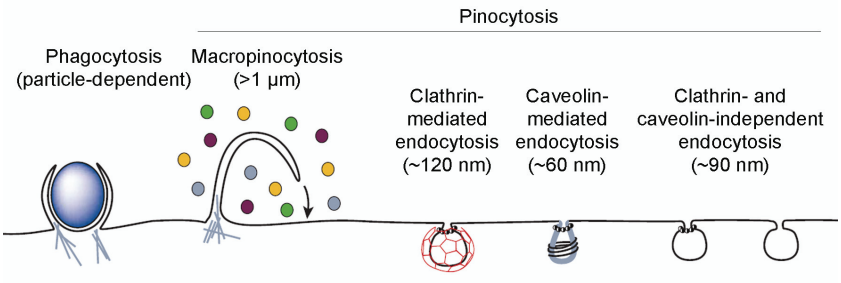


**Figure 7. The different pathways of endocytosis.** The different pathways of endocytosis can be divided into two main classes, phagocytosis and pinocytosis. Figure is taken from (Conner et al. 2003a) and figure legend is modified.

### 1.5.1 CLATHRIN-MEDIATED ENDOCYTOSIS

CME occurs constitutively in all mammalian cells and is crucial for communication between cells during tissue and organ development (Di Fiore et al. 2001; Seto et al. 2002). CME is important for the synaptic transmission in neurons (De Camilli et al. 1996), and by downregulating proteins in neurons it may also control the strength of the synaptic transmission and thereby play a role in learning and memory (Beattie et al. 2000; Traub 2003). CME is also important for various kinds of receptor-mediated endocytosis, including endocytosis of the EGFR (Figure 8). Clathrin assembles into a trimeric structure, and each clathrin triskelion contains three heavy chains (CHC) and three light chains (CLC) (Figure 8 A) (reviewed in Edeling et al. 2006). This three-legged unit is able to form a lattice with other clathrin triskeliae. The domain at the end of each leg can interact with adaptor proteins needed for endocytosis. Adaptor proteins form a link between the membrane, clathrin and the proteins that are to be transported into the clathrin coat (reviewed in Traub 2003).

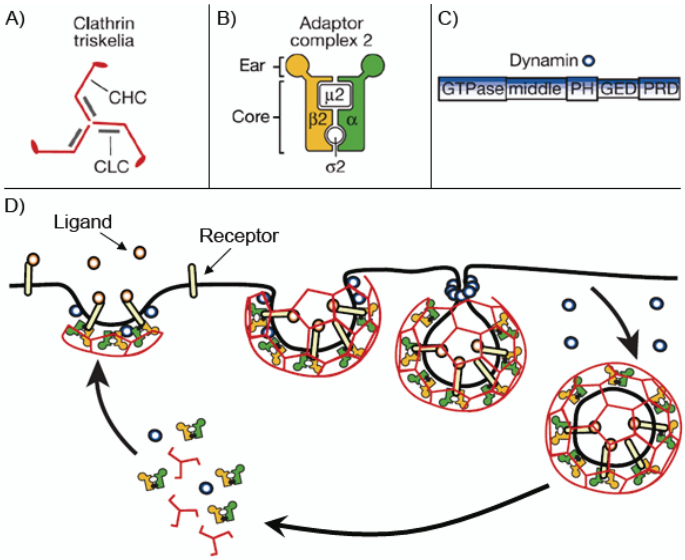


**Figure 8. Clathrin-mediated endocytosis.** (A-C) The main components of the clathrin-coated pit are clathrin, AP2 and dynamin. See text for details. (D) The receptor is transported into the CCP and the membrane is invaginated. After pinching off, the components of the clathrin coat disassociate and are reused for subsequent internalization steps. The figure is from (Conner et al. 2003a), and both figure and figure legend is modified.

The main adaptor protein complex involved in CME is AP2. AP1, AP3 and AP4 are also involved in clathrin coat formation, but they are important for the sorting events at endosomes and TGN rather than at the plasma membrane. AP2 has four structural subunits, α, β2, μ2, and σ2 (Figure 8B) and interacts with clathrin (through its β2 subunit), PIs (through its α-subunit) and with cargo (through its μ2-subunit) (Kirchhausen et al. 1997; Praefcke et al. 2004). The binding to cargo occurs through distinct AP2-binding sorting signals (section 1.5.3). The two large subunits of AP2, α and β2, are each composed of two domains linked through a flexible hinge region, the N-terminal core and the C-terminal ear. The ear of the AP2 α-subunit has been shown to interact with several other proteins, some of which also interact with clathrin and serve as adaptor proteins themselves. Examples are Dab2, CALM, AP180, epsin and Eps15 (EGFR-pathway substrate-15) (reviewed in Traub 2003).

Epsin is localized to clathrin-coated pits (Stang et al. 2004) and binds to the membrane through its ENTH domain which interacts with PIP2 (Ford et al. 2002; Itoh et al. 2005). Epsin also interacts with clathrin and AP2 and has been proposed to function as an adaptor protein (reviewed in Wendland 2002). Epsin has also been reported to mediate membrane curvature (Ford et al. 2002). In addition it contains multiple ubiquitin interacting motifs (UIMs), and it has therefore been suggested that epsin may function to sort ubiquitinated cargo for clathrin-mediated endocytosis (Barriere et al. 2006; Duncan et al. 2006; Hawryluk et al. 2006). Epsin interacts with the Eps15 homology (EH) domain of Eps15. Eps15 is also localized to the clathrin-coated pit and additionally interacts with AP2 and ubiquitin. The role of Eps15 in endocytosis is not clear, but it is thought to act together with epsin to sort ubiquitinated cargo into the clathrin coat (Chen et al. 1998; Hawryluk et al. 2006). The importance of Eps15 and AP2 for endocytosis of EGFR has been studied by overexpression of Eps15 with a mutated EH-domain (Eps15EH29). Eps15EH29 do not localize to the plasma membrane and also sequesters AP2 from its plasma membrane localization (Benmerah et al. 1999; Benmerah et al. 2000). Removal of AP2 dissolves the existing clathrin coats and by this approach it is found that upon EGF-incubation, new clathrin-coated pits are formed at the plasma membrane (Johannessen et al. 2006). These coats are thus formed in the absence of AP2 and Eps15, and this indicates that these two proteins are not essential for EGFR endocytosis. In contrast, Grb2, which is also involved in signaling from EGFR (section 1.4.1), is important for endocytosis of EGFR (Jiang et al. 2003a; Huang et al. 2004; Huang et al. 2005), possibly because of its role in recruitment of EGFR into clathrin-coated pits (Stang et al. 2004). Grb2 is recruited to the EGF-induced clathrin coats and confirms the importance of this protein for endocytosis of EGFR (Johannessen et al. 2006).

Dynamin is a protein required for CME, but it is also required for caveolin-mediated endocytosis and some clathrin- and caveolin-independent endocytic pathways (Hinshaw 2000; Sever et al. 2000). Dynamin contains a pleckstrin homology (PH) domain that can interact with PIP2 in the plasma membrane (Figure 8C). By interaction between individual dynamin molecules (through domains like the GTPase effector domain) and with other endocytic proteins (through prolin rich sequences), dynamin forms rings

around an invaginated membrane to mediate its pinching off (Figure 8D) (reviewed in Conner et al. 2003a).

### 1.5.2 CAVEOLIN-MEDIATED ENDOCYTOSIS

Caveolae have been suggested to be involved in constitutive endocytosis in endothelial cells (Henley et al. 1998; Oh et al. 1998) and in endocytosis of glycosyl-phosphatidylinositol (GPI) -anchored proteins and cross-linked gangliosides like GM1 (Parton et al. 1994; Kurzchalia et al. 1999). However, there are contradicting reports saying that caveolae are immobile structures (Thomsen et al. 2002; Pelkmans et al. 2005; Kazazic et al. 2006) and that internalization of GPI-anchored proteins is very slow (Johannes et al. 2002). Endocytosis via caveolae can, however, be triggered for example by simian virus 40 (Pelkmans et al. 2001; Pelkmans et al. 2002; Tagawa et al. 2005), cross-linking of membrane receptors (Hommelgaard et al. 2005), or incubation with the phosphatase inhibitor okadaic acid (Parton et al. 1994). It is reported that EGFR can be internalized through caveolae (Sigismund et al. 2005), but this finding is contradicted in a report showing that EGF does not trigger mobilization of caveolae (Kazazic et al. 2006).

### 1.5.3 SIGNALS FOR ENDOCYTOSIS

Transmembrane proteins often carry an endocytic signal localized in the intracellular part of the protein. These signals are often di-leucine-based or tyrosine-based (Heilker et al. 1999; Bonifacino et al. 2003; Traub 2003). These motifs are recognized by endocytic adaptor proteins that guide their movement into clathrin-coated pits for internalization. An example of a common tyrosine-based motif is the YXXΦ motif (where X is any amino acid and Φ is a bulky, hydrophobic amino acid). All four of the adaptor complexes (AP-1 to AP-4) bind this motif through their μ subunit (Robinson 2004). The requirement of such a motif for internalization varies for different proteins, but for the transferrin receptor the motif is shown to be required for its internalization (Motley et al. 2003). Also the EGFR contains an AP2-interacting YXXΦ motif (Nesterov et al. 1995a; Sorkin et al. 1996; Huang et al. 2003) as well as a di-leucine motif that can interact with AP2 (Huang et al. 2003). The role of the interaction between EGFR and AP2 is debated, but it seems that the interaction may facilitate, but is not

required for endocytosis of EGFR (Nesterov et al. 1995b; Conner et al. 2003b; Motley et al. 2003; Huang et al. 2004). Thus, AP2 plays a more important role for internalization of the transferrin receptor than for the EGFR (Johannessen et al. 2006). Additional signals such as ubiquitination may be of importance for internalization of EGFR.

### 1.5.4 UBIQUITIN IN ENDOCYTOSIS

Upon activation of the EGFR, Cbl is recruited to the EGFR either directly through the Cbl PTB domain or indirectly through binding of Grb2 (Levkowitz et al. 1996; Waterman et al. 2002). Upon binding to EGFR, directly or indirectly, Cbl is activated and mediates ubiquitination of lysine residues of the EGFR by multiple monoubiquitination or by polyubiquitination (Levkowitz et al. 1999; Huang et al. 2006). It has been suggested that ubiquitination may serve as a signal for endocytosis of EGFR (Levkowitz et al. 1998; Miyake et al. 1998; Stang et al. 2004) but whether this involves mono-, multiple mono- or polyubiquitination is currently not clear as there are indications in different directions (Raiborg et al. 2002b; Haglund et al. 2003; Hawryluk et al. 2006).

Several reports have shown that reduced ubiquitination inhibits EGFR endocytosis. Upon overexpression of the Cbl-and ubiquitin-interacting protein T-cell ubiquitin ligand (TULA), the ligand-induced endocytosis and degradation of EGFR is impaired (Feshchenko et al. 2004; Kowanetz et al. 2004). The mechanism behind this inhibition by TULA, and thus the role of ubiquitination in EGFR endocytosis, was further investigated in Paper IV. Likewise, expressing different mutant forms of Cbl and overexpression of the Cbl-binding protein Sprouty have been reported to inhibit ligand-induced ubiquitination and endocytosis of the EGFR (Thien et al. 2001; Wong et al. 2002; Fong et al. 2003; Stang et al. 2004). It seems that ubiquitination is necessary for endocytosis of EGFR, but whether EGFR itself needs to be ubiquitinated is a matter of debate. Studies claiming that ubiquitination of EGFR is not required can been questioned because the EGFR ubiquitination have not been completely abolished. Even a low level of ubiquitination could be sufficient to serve as an internalization signal (Shih et al. 2000).

Although several reports indicate that inhibited or increased ubiquitination affects degradation and not primarily endocytosis of the EGFR (Levkowitz et al. 1998; Duan et al. 2003; Jiang et al. 2003b), there are also indications that ubiquitination of EGFR is important for its endocytosis. First, it seems that ubiquitination plays a role at the plasma membrane, as it is shown that EGF-induced ubiquitination of EGFR occurs at the plasma membrane (Stang et al. 2000). Second, it has been suggested that the requirement for Grb2 in EGFR endocytosis (Sorkina et al. 2002; Huang et al. 2004; Huang et al. 2005) is due to its recruitment of Cbl to EGFR (Waterman et al. 2002; Jiang et al. 2003a). Additionally, overexpression of a mutant form of Grb2, not able to bind Cbl, inhibits recruitment of EGFR to coated pits (Stang et al. 2004). Third, it has been demonstrated that fusion of ubiquitin to a truncated form of EGFR mediates constitutive endocytosis (Haglund et al. 2003; Mosesson et al. 2003). Fourth, there are indications that Eps15 and epsin, which are proteins harbouring UIMs and localize to the clathrin coat, may function as endocytic adaptor proteins (Stang et al. 2000; Traub 2003; Barriere et al. 2006; Hawryluk et al. 2006). Most recently, however, it was reported that mutation of several lysines abolishing EGFR ubiquitination does not inhibit endocytosis when the EGFR kinase activity is intact, but does inhibit endocytosis when the kinase activity is impaired (Huang et al. 2007).

## 1.6 ENDOSOMAL SORTING OF ERBB PROTEINS

### 1.6.1 THE ENDOSOMAL SORTING MACHINERY

The sorting of ubiquitinated cargo, including EGFR, on sorting endosomes depends on several different proteins. Hepatocyte growth factor regulated tyrosine kinase (Hrs) has been proposed to initiate the sorting process (reviewed in Raiborg et al. 2002b). Hrs localizes to the endosomal membrane through its FYVE and coil-coil domains and it can recruit clathrin in a PI(3)P dependent manner (Raiborg et al. 2001). Hrs also interacts with Eps15 and signal-transducing adaptor molecule (STAM) (Asao et al. 1997; Bean et al. 2000) and these three proteins can interact with ubiquitin through their UIM domains (Polo et al. 2002). The complex of Hrs/Eps15/STAM is suggested to cooperate in binding and concentration of ubiquitinated cargo while the non-ubiquitinated proteins will not be retained (Raiborg et al. 2002b). Hrs further interacts

with a component of the endosomal sorting complex required for sorting-I (ESCRT-I) (Katzmann et al. 2001). Hrs may therefore also be able to indirectly recruit the rest of the sorting machinery, ESCRT-II (Babst et al. 2002b) and ESCRT-III (Babst et al. 2002a).

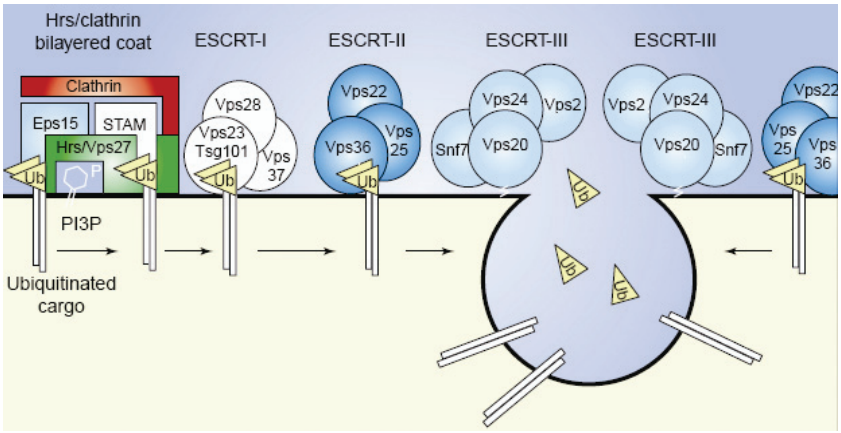


**Figure 9. Endosomal sorting machinery.** Sorting is initiated by Hrs which together with STAM and Eps15 can recognize ubiquitinated cargo. Hrs can recruit clathrin and also Vps23 of the ESCRT-I complex. The ESCRT-II and ESCRT-III complexes are then recruited eventually mediating invagination and scission of the membrane including the cargo. The figure is from (Raiborg et al. 2003) and figure legend is modified.

### 1.6.2 SORTING OF EGFR

The transport and sorting on intracellular organelles are also dependent on sorting signals and ubiquitination, but the pattern of ubiquitination and the sequence of the sorting signals may be different compared to translocation at the plasma membrane. Cbl-mediated ubiquitination is shown to be important for sorting of EGFR to inner vesicles of MVBs (Levkowitz et al. 1999; Longva et al. 2002; Duan et al. 2003; Grovdal et al. 2004). In contrast, endocytosed receptors that are not ubiquitinated are not internalized into vesicles of MVBs and are in stead recycled to the plasma membrane (Raiborg et al. 2002a), which is the case for the transferrin receptor (Dautry-Varsat et al. 1983) and TGF-α activated EGFR (Figure 10) (Ebner et al. 1991; French et al. 1995; Longva et al. 2002; Alwan et al. 2003). Deubiquitinating enzymes are also

important in this process in that they can prevent ubiquitin-mediated degradation of proteins. It has been shown that the deubiquitinating enzyme UBPY (also called Usp8) can prevent degradation of the EGFR by deubiquitinating the EGFR on endosomes (Mizuno et al. 2005). A maturation of the MVB and the fusion with a lysosome is the end of the journey for EGFR (Figure 10) (Futter et al. 1996). In the lysosomes the receptor is degraded by lysosomal proteases (Authier et al. 1999) and the attenuation of signaling is fulfilled.

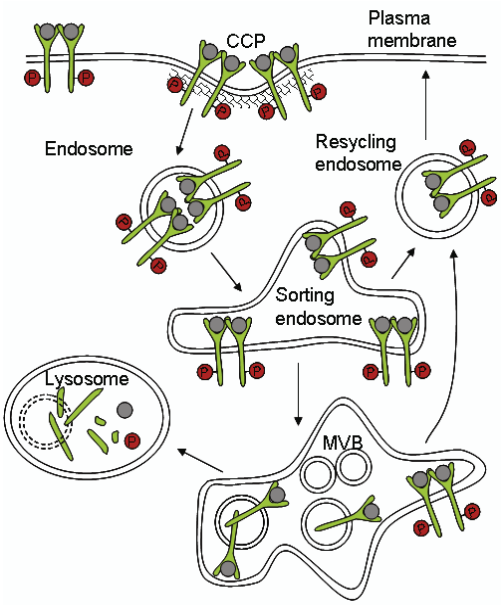


**Figure 10. Trafficking of EGFR.** The receptors are taken in through clathrin-coated pits. The forming vesicles fuse to endosomes. In the sorting endosome, the receptors that are to be recycled are transferred to the recycling endosomes while the receptors that are to be degraded are internalized to inner vesicles of an MVB. In the lysosomes, the receptors are eventually degraded. The figure is based on (Carpenter 2000).

### 1.6.3 SORTING OF ERBB2

Whether the mechanism of sorting of ErbB2 and ErbB3 is similar to EGFR is not known. Although it is reported that ErbB2 is endocytosis resistant (section 1.1.2), others have shown that it recycles (Austin et al. 2004). Recycling is also reported for ErbB3 (Waterman et al. 1998). However, these results is debated as others have found that both ErbB2 (upon GA treatment) and ErbB3 are rapidly degraded upon internalization (Mimnaugh et al. 1996; Xu et al. 2001; Qiu et al. 2002; Zhou et al. 2003). CHIP-mediated ubiquitination and downregulation of ErbB2 can be induced by incubation with GA, but whether this ubiquitination serves as a signal for endocytosis or sorting is not known. Ubiquitination of ErbB2 by CHIP is reported to result in proteasomal degradation of ErbB2 (Mimnaugh et al. 1996), but others have shown that ErbB2 is degraded in lysosomes (Austin et al. 2004). The same report also suggested that GA

only affects the rate of endosomal sorting and not internalization of ErbB2 (Austin et al. 2004). It has been reported that GA induces internalization of full length ErbB2 which is degraded in lysosomes, and that the process is proteasome dependent (Lerdrup et al. 2006). Other reports say that a cleavage is important before internalization and degradation, and possibly the fragments may be degraded by different processes (Tikhomirov et al. 2000; Tikhomirov et al. 2001; Tikhomirov et al. 2003; Lerdrup et al. 2007). Recent findings in our group show that the GA-induced endocytosis is not dependent on proteasomal activity, while the sorting for inner vesicles of MVBs is (Pedersen et al. In press)

# 2 AIMS OF THE STUDY

When an ErbB protein is activated, it transmits signals to the interior of the cell. A major way to attenuate signaling is to downregulate the receptor by endocytosis followed by degradation. The expression pattern of the different ErbB proteins varies between different cancers and possibly also within one tumor and is important for treatment, survival and prognosis. Furthermore, to know how the receptors cooperate is important. The main aim of this project has therefore been **to investigate endocytic mechanisms of the ErbB proteins** and to understand what impact they have on the endocytosis of each other.

It is known that ErbB2 escapes downregulation, but there are contradicting reports on whether this is caused by inhibition of endocytosis or caused by a rapid recycling upon endocytosis. It is also known that ErbB2 has an inhibitory effect on the downregulation of EGFR but the reason for this is unclear. Previous publications in our group had concluded that ErbB2 was endocytosis deficient. Our first aim was therefore **to investigate the mechanisms responsible for the inhibited endocytosis of ErbB2** and thus how ErbB2 inhibits endocytosis of EGFR.

We had seen that ErbB2 inhibited endocytosis of the EGFR. Since ErbB3 was also known to be endocytosis impaired and display inefficient ligand-mediated endocytosis, our second aim was **to investigate whether ErbB3 inhibits endocytosis of the EGFR** in a similar manner as ErbB2. We also wanted to study trafficking of ErbB3 and the mechanism controlling its subcellular localization.

Since ErbB2 is endocytosis resistant it is difficult to study its trafficking. GA is used to induce downregulation of ErbB2 due to the ability of GA to counteract the stabilizing function of Hsp90 on ErbB2. Since it was unclear whether GA-induced downregulation of ErbB2 was affected by the other ErbB proteins, our third aim was **to investigate whether GA can induce endocytosis of ErbB2 in cells only expressing ErbB2**. By comparing this possible GA-induced endocytosis of ErbB2 with cells also expressing

EGFR and/or ErbB3, we wanted to study the possible implication of other ErbB proteins.

The importance of ubiquitination for endocytosis of EGFR is debated. Some studies have concluded that ubiquitination of EGFR is of little importance for endocytosis while other studies have concluded differently. Our fourth aim was **to investigate whether ubiquitination was important for endocytosis of EGFR** by overexpressing the protein TULA, which is known to inhibit ubiquitination of EGFR by interacting with Cbl.

# 3 SUMMARY OF PAPERS

## 3.1 PAPER I

*The inhibitory effect of ErbB2 on epidermal growth factor induced formation of clathrin-coated pits correlates with retention of epidermal growth factor receptor-ErbB2 oliomeric complexes at the plasma membrane.* Camilla Haslekås[1], **Kamilla Breen**[1], Ketil W. Pedersen, Lene E. Johannessen, Espen Stang and Inger Helene Madshus.

It has been demonstrated that ErbB2 is endocytosis resistant and that ErbB2 also inhibits endocytosis of EGFR in heterodimers. The reason for the inhibition of EGFR endocytosis is not understood, and by comparing non-isogenic cell lines different results have been obtained. Using isogenic cell lines of porcine aortic endothelial (PAE) cells stably transfected with EGFR and ErbB2, we show that upon expressing increasing levels of ErbB2, the cells internalized decreasing amounts of EGF. By using immuno-EM, we found that in cells expressing high levels of ErbB2, the EGFR was retained at the plasma membrane after EGF-stimulation. Consistently, there was no increase in intracellularly localized EGFR after EGF-stimulation in cells expressing a high level of ErbB2. We exclude three possible explanations for the ErbB2 mediated inhibition of EGFR-endocytosis. First, we found that there was no increase in recycling of EGF in cells co-expressing ErbB2. Second, the activation of EGFR was found to be similar in cells with and without ErbB2. Third, ErbB2 is not retained at the plasma membrane through its interaction with the protein Erbin, which is suggested to be involved in membrane localization. By counting clathrin-coated pits at the plasma membrane, we found that while cells expressing only EGFR induced new clathrin-coated pits upon EGF-incubation, cells co-expressing EGFR and ErbB2 did not induce formation of new clathrin-coated pits. This finding can explain why EGFR-ErbB2 dimers are endocytosis resistant.

## 3.2 PAPER II

*ErbB3 is constitutively endocytosed in a clathrin dependent manner*. **Kamilla Breen**, Sissel Beate Rønning, Nina Marie Pedersen, Espen Stang and Inger Helene Madshus.

We and others have shown that overexpression of ErbB2 inhibits endocytosis of EGFR. Also ErbB3 has been reported to be endocytosis impaired because of the slow internalization rate of heregulin-bound ErbB3 compared to EGF-bound EGFR. We therefore wanted to investigate whether ErbB3 had a similar effect as ErbB2 on the endocytosis of EGFR. By using three stably transfected cell lines expressing EGFR, EGFR/ErbB2 or EGFR/ErbB3, we found that even though ErbB3 existed in active heterodimers with EGFR, the endocytosis rate of EGF was only inhibited in cells expressing EGFR and ErbB2. By additionally studying the EGF-induced downregulation of surface-localized EGFR by flow-cytometry, we conclude that the presence of ErbB3 does not inhibit downregulation of EGFR. By studying the sub-cellular localization of ErbB3, we found that it was localized at the plasma membrane as expected, but also that a significant fraction localized intracellularly. Surprisingly, by incubating cells with an ErbB3 antibody and subsequently chase the antibody, we found that the main part of the intracellularly localized ErbB3 was a result of constitutive endocytosis of ErbB3. By overexpressing dominant negative dynamin or by knocking down clathrin heavy chain with siRNA, we observed a clear inhibition of the constitutive endocytosis of ErbB3. We conclude that ErbB3 can not inhibit endocytosis of EGFR because it is itself constitutively endocytosed in a dynamin- and clathrin-dependent manner.

## 3.3 PAPER III

*Expression of EGFR or ErbB3 facilitates Geldanamycin-induced downregulation of ErbB2.* Nina Marie Pedersen[1], **Kamilla Breen**[1], Camilla Haslekås, Espen Stang and Inger Helene Madshus.

Although ErbB2 is normally endocytosis deficient, its endocytosis can be induced by incubating cells with GA. GA, and derivates of GA, are shown to inhibit growth of

cancer cells that overexpress ErbB2. Furthermore, GA is used experimentally to study mechanisms involved in downregulation of ErbB2. To investigate a possible role of other ErbB proteins in GA-induced endocytosis of ErbB2, we first wanted to investigate whether this endocytosis of ErbB2 could be induced when ErbB2 was in a homodimeric/-oligomeric form. By using stably transfected cell lines expressing only ErbB2, we found that GA-induced downregulation of ErbB2 can occur in cells expressing ErbB2 only. However, in cells additionally expressing EGFR and/or ErbB3 the GA-induced downregulation of ErbB2 was significantly increased. We show that this increase is not caused by a GA-induced downregulation of EGFR or ErbB3, or by GA-induced phosphorylation of any of the receptors. GA affects the stabilizing function of Hsp90 on ErbB2. Hsp90 is a dimeric molecule, and can potentially form a tight interaction between ErbB2 molecules and stabilize homodimers/-oligomers more than heterodimers/-oligomers. We found, however, that the initial rate of GA-induced dissolving of ErbB2 homo-oligomers was the same for ErbB2 containing hetero-oligomers.

## 3.4 PAPER IV

*The Cbl-interacting protein TULA inhibits dynamin-dependent endocytosis*. Vibeke Bertelsen, **Kamilla Breen**, Kirsten Sandvig, Espen Stang and Inger Helene Madshus.

It is known that the T-cell ubiquitin ligand, TULA, interacts with Cbl and inhibits ubiquitination of the EGFR. The block in ubiquitination has been suggested to explain the TULA-mediated inhibition of EGFR endocytosis. We wanted to investigate the role of TULA in EGFR endocytosis in more detail. Surprisingly, we found that in addition to inhibiting endocytosis of EGFR, TULA overexpression also inhibited the endocytosis of several other proteins such as the transferrin receptor, the low density lipoprotein (LDL) receptor, the major histocompatibility complex-I (MHC-I) and CD59. With the endocytosis of such a wide range of proteins being inhibited, we speculated that a protein involved in several forms of endocytosis was affected by overexpression of TULA. One such protein is dynamin. We found that endocytosis of all proteins affected by TULA overexpression was also inhibited upon transfection with a dominant negative

form of dynamin; their endocytosis is thus dynamin dependent. A protein known not to depend on dynamin for endocytosis is the plant toxin ricin. Ricin was, as expected, not affected by overexpression of TULA. We also found that the TULA-mediated block in endocytosis could be rescued upon overexpression of wt dynamin. Consistently, the endocytosis was not inhibited by overexpression of an SH3-mutant of TULA unable to bind dynamin (W279L-TULA). In the case of the EGFR, however, the TULA-mediated inhibition of ubiquitination and endocytosis was clear both upon co-transfection with TULA and wt dynamin and upon transfection with W289L-TULA. This indicates that there is a correlation between inhibited ligand-induced ubiquitination of EGFR and inhibition of endocytosis of EGFR.

# 4 METHODOLOGICAL CONSIDERATIONS

## 4.1 EXPERIMENTAL MODEL SYSTEM

Most of the experiments included in this work have been done using PAE cells. The PAE cells do not express endogenous ErbB proteins, but were stably transfected with different members of the EGFR family, EGFR, ErbB2 and/or ErbB3. When testing how the receptors influence each other, it is an advantage of PAE cells that there is no background of endogenous ErbB proteins. Additionally, the expression level of the receptors is controlled by this approach. For example, when a cell line expressing ErbB2 is further transfected with EGFR, the new cell line will have the same level of ErbB2 as the original cell line. In Paper III, the expression of ErbB2 in the different cell lines is therefore equal, and different ErbB2 expression can be excluded as a reason for the different rate of GA-induced endocytosis of ErbB2. An additional advantage of the PAE cells is that they are large and have a flattened shape. This makes them convenient for microscopy as each cell has a relatively large cytosol for investigation. SKBr3-cells are, in contrast to PAE cells, small and circularly shaped. In addition, SKBr3-cells express ErbB3 in too low amounts to be detected by the available ErbB3 antibody and confocal imaging upon immunostaining (Paper II). It may be argued, however, that the PAE cell-lines used are artificial and that they do not represent the situation in cells endogenously expressing these receptors. A cell line that originally does not express any of the ErbB proteins may lack some of the control systems needed to regulate the receptors. However, we have thoroughly tested the localization, dimerization, endocytosis and downregulation of the receptors. Nothing implies that there are control mechanisms missing. When possible, we confirmed the results obtained in PAE-cells using SKBr3 cells.

However, a disadvantage of the stably transfected PAE cell lines was that expression of receptors was not constant over time. For unknown reasons, after a limited number of passages, some cells reduced or stopped the expression of the transgene, although the antibiotic resistance was still intact. For this reason cells were never grown confluent and were kept in culture only for a limited number of passages. The expression levels of

receptors were regularly tested by flow cytometry and heterogenic cell populations were not used for experiments.

## 4.2 ANTIBODY SPECIFICITY

Many of the methods used depend on antigen-antibody interaction. It is therefore crucial that the antibodies are specific. By studying a band on a Western blot, it can be concluded whether the antibody recognizes a protein of the correct size, and this is an indication of specificity. However, in case of the ErbB proteins, their similar molecular weights make it difficult to distinguish the receptors based on size. The use of the different PAE cell lines was therefore an important tool for testing the ErbB-antibodies. Despite the specificity claimed by the manufacturers, several of the antibodies tested cross-reacted with other members of the ErbB family, and some antibodies also showed reactivity in cells not expressing any of the receptors. These antibodies were of course excluded. Specificity was in some cases additionally confirmed upon transient transfection with the plasmid encoding the target protein. The different PAE cell lines also allowed flow-cytometry and immunocytochemical staining to be used for determining antibody specificity. If the antibody bound non-specifically, resulting in a high background staining, it was not used for techniques like flow-cytometry or immunofluorescence.

When working with live cells an additional issue to consider, when using antibodies to proteins at the cell surface, is the possibility of antibody-induced internalization of the protein and/or activation of receptors. Several antibodies have this effect. In Paper IV we took advantage of this, using antibody against MHC-I and DC59 to induce their internalization. When investigating endocytosis of ErbB3, however, it was important that the anti-ErbB3 antibody did not induce endocytosis, as this would disqualify our conclusion about constitutive endocytosis of ErbB3. The product sheet following the antibody contained a reference to a study showing that the antibody did not induce endocytosis or activation of ErbB3 (Chen et al. 1996). To additionally confirm this, we tested the surface expression of ErbB3 by flow-cytometry after incubation with this ErbB3 antibody. As expected, there was no induction of endocytosis.

## 4.3 FLOW-CYTOMETRY

When measuring the expression level of receptors at the plasma membrane of the cells, we exclusively used antibodies against extracellular epitopes. This was done to avoid the risk of non-specific binding, which is increased upon permeabilization of cells. Permeabilization was avoided also because it gives access to intracellularly localized receptors that would influence the results when studying surface localized proteins. When investigating downregulation of receptors from the plasma membrane, it is important to have saturating concentrations of the antibody. If the epitopes are not saturated with antibody, variation in pipetting can affect the fluorescence intensity without reflecting a difference in epitope accessibility. We therefore optimized the amount of each antibody to obtain saturation.

## 4.4 CONFOCAL AND IMMUNO ELECTRON MICROSCOPY

Microscopy, both confocal microscopy and immuno electron microscopy, was used throughout this study. When investigating single cells in a microscope, individual cell variations can easily be misinterpreted, and inclusion of a high number of cells is therefore important. For this reason, quantifications were made. The quantification generalizes the results, and by using standard deviations illustrates cellular variation. When EM-results were quantified, sectioned cells were chosen in a systematic random fashion, reducing the problem of cell variations. Each quantification experiment was performed on a minimum of three separate grids. Confocal microscopy can also be used to compare fluorescence intensity, although some pitfalls should be considered. When comparing data from different samples, the labelling conditions and microscope settings should be the same for all samples. There is also a risk of bleaching the fluorochrome and pictures should be taken before the sample is significantly bleached. As this method is not very sensitive, it is not well suited for quantification. Quantifications were therefore only used when a clear difference was observed, such as when studying internalization of EGF in cells expressing or not expressing ErbB2 (Paper I).

## 4.5 QUANTIFYING WESTERN BLOTS

Western blotting is a semi-quantitative method, and quantification should be done with precaution. However, Western blotting is often the best or only way to obtain quantitative data. Depending on the quality of the Western blots, different background settings are needed, and depending on the background settings, it may be crucial to make the regions of interest (ROIs) the same size. Values obtained can vary significantly depending on these settings. Incorrect values can also be the result if the pixels are saturated or if there is too much protein in the membrane, leading to complete consumption of substrate and thus reduced signal. All quantifications have therefore been made with care, and always compared with the visual image. All values were adjusted according to a loading control, and all ROIs compared with each other were of the same size.

## 4.6 DOWNREGULATION OF PROTEINS USING SIRNA

The use of RNA interference by short interfering RNA (siRNA) is a relatively new method for downregulation of proteins in biomedical research. The biological phenomenon of siRNA sequences in post-translational gene silencing was discovered in 1999 by Hamilton and coworkers (Hamilton et al. 1999), and synthetic siRNA was shortly thereafter shown to induce gene silencing in mammalian cells (Elbashir et al. 2001). A problem with siRNA is off-target effects. The introduced siRNA may bind to mRNAs with similar sequences as the target mRNA, leading to problems interpreting the results, and to potential toxicity. By using thoroughly tested target sequences that do not bind to other mRNAs, the off-targeting effect can be minimized. An additional approach is to use several different sequences in parallel and to verify that they all give the same result. By introduction of too much RNA, the cell may misinterpret the RNA as a viral product and induce an immune response. By always including the same amount of a control sequence in parallel in the assay, the possible effect of an immune response and the potential toxicity of the transfection reagent can be monitored in the control cells.

## 4.7 TRANSIENT TRANSFECTION AND OVEREXPRESSION OF PROTEINS

Transient transfection of cells to overexpress a protein of interest is an important tool to study interaction partners, function and localization of gene products of interest. When highly overexpressed, however, proteins may aggregate and/or have a different localization than when they are moderately expressed. Interpretation concerning localization under such conditions should therefore be avoided. Upon overexpression, an observed phenotype may be the result of a function of the overexpressed protein, but may also be the result of endogenous proteins being sequestered by the overexpressed protein. However, upon overexpression of TULA (Paper IV), we took advantage of the ability of TULA to sequester Cbl in order to study the effect of Cbl-depletion on endocytosis of EGFR.

## 4.8 BIOTINYLATION ASSAY

Biotinylation of plasma membrane proteins is a convenient way to study their internalization, and was used in Paper II to study internalization of ErbB3. In contrast to flow cytometry, where endocytosis is studied indirectly by the decrease in protein amount at the plasma membrane, the biotinylation assay detects internalized proteins directly by precipitation of the biotin-labelled internalized proteins. The biotinylation assay also has the advantage that cell surface proteins are labelled with a biotin-molecule bound to a monovalent reactive group, and therefore no clustering of proteins will be induced. Clustering can happen with antibodies or di- or polyvalent cross-linkers. The amount of internalized protein is compared with two controls. A negative control contains cells that are not chased and represents the low background level of biotin left on the cell surface after the stripping procedure. To minimize the background, the stripping or reduction was done thoroughly and three times. The positive control contains cells that have not been stripped and represents the total amount of biotin-labelled protein. Since these two controls are always included, there is no risk that retaining surface-localized protein interferes with the result.

# 5 DISCUSSION

The papers included in this thesis contribute new information concerning endocytosis of ErbB proteins. Endocytosis of ErbB proteins is a vast and complicated field of biology and a large amount of new knowledge is continuously being reported. The fact that contradictory publications are frequently generated makes the field even more complicated to understand. Contradictory publications are probably mainly a result of differences in experimental setup and use of different reagents and cell lines.

## 5.1 IMPAIRED DOWNREGULATION OF ERBB2

### 5.1.1 IMPAIRED ENDOCYTOSIS VERSUS RAPID RECYCLING

In Paper I we show that the downregulation of ErbB2 is impaired. Impaired downregulation could be a result of impaired endocytosis or of rapid recycling upon endocytosis. It has been reported that ErbB2, instead of being endocytosis impaired, is efficiently recycled (Austin et al. 2004), and further that overexpression of ErbB2 also causes increased recycling of EGF and EGFR (Lenferink et al. 1998; Worthylake et al. 1999; Hendriks et al. 2003). However, although ErbB2 increased the rate of EGFR recycling, the main inhibitory effect of ErbB2 on EGFR endocytosis was by Hendriks and colleagues judged to be caused by the impaired endocytosis of ErbB2 (Hendriks et al. 2003). Several other studies have also concluded that ErbB2 is endocytosis resistant (Sorkin et al. 1993; Wang et al. 1999; Hommelgaard et al. 2004; Longva et al. 2005; Lerdrup et al. 2006). In Paper I, we confirmed that ErbB2 was endocytosis resistant and further showed that its heterodimerization with the EGFR also inhibited ligand-induced downregulation of the EGFR. The inhibited downregulation of EGFR in cells overexpressing ErbB2 was not due to rapid recycling of EGFR/ErbB2 complexes, as incubation of cells with monensin did not cause accumulation of EGFR or ErbB2 intracellularly. We therefore conclude that ErbB2 is an endocytosis resistant receptor.

### 5.1.2 IMPAIRED ENDOCYTOSIS OF ERBB2

The reason why ErbB2 is endocytosis resistant is still unclear. There are indications that the endocytosis resistance of ErbB2 is a result of its localization to lipid rafts on membrane protrusions and to other areas outside clathrin-coated pits (Hommelgaard et al. 2004; Offterdinger et al. 2007), and that signals for endocytosis are missing in its cytoplasmic tail (Sorkin et al. 1993). In addition to what we showed in Paper I, several previous studies have shown that the presence of ErbB2 affects downregulation of EGFR (Muthuswamy et al. 1999; Wang et al. 1999; Worthylake et al. 1999). We found that EGF-induced clathrin-coated pits were absent in cells co-expressing EGFR and ErbB2 (Paper I), but since the detailed mechanisms in the formation of clathrin-coated pits is not understood, it is difficult to investigate what differentiates ErbB2 from EGFR in this respect. It is, for example, still not known whether EGF-induced clathrin-coated pits are formed around the activated receptor, or if the receptor moves into a preformed coated pit formed in another area of the plasma membrane. One possible explanation for the endocytosis resistance of ErbB2 could be that binding of ErbB2 to a specific protein or lipid may inhibit formation of clathrin-coated pits or alternatively restrain ErbB2 to certain areas of the plasma membrane, and inhibit its translocation into clathrin-coated pits. One protein that interacts with the C-terminal part of ErbB2 (Borg et al. 2000) and is suggested to affect endocytosis of ErbB2 is Erbin (Jaulin-Bastard et al. 2001; Birrane et al. 2003). However, as we showed in Paper I, interaction with Erbin cannot explain the endocytosis resistance of ErbB2. Our data demonstrates that an ErbB2 mutant, which lacks the Erbin interacting domain, also was endocytosis resistant. Hsp90 may be a protein that directly inhibits endocytosis. It is reported that Hsp90 inhibits ErbB2 heterodimerization and that Hsp90 dissociates from ErbB2 following ligand-induced heterodimerization (Citri et al. 2004). The loss of Hsp90 has been reported to result in increased kinase activity of ErbB2 (Citri et al. 2004) and has also been reported to increase Src-mediated phosphorylation of ErbB2 (Xu et al. 2007). The binding of Hsp90 may thus also inhibit ubiquitination and/or other modifications of ErbB2 (discussed in section 5.3).

Although the lack of a modification could explain why ErbB2 does not localize to the clathrin coat, it does not explain why the EGFR in an EGFR/ErbB2 heterodimer can not

recruit the proteins needed for coat formation and/or localization. Therefore, it seems likely that a protein needed for the formation of clathrin-coated pits, or needed for the recruitment of EGFR into such coated pits, is not recruited to the EGFR/ErbB2 heterodimer. Either such a protein needs to be recruited to both receptors within the dimer in order to properly serve its function, or ErbB2 somehow inhibits the recruitment of the protein to EGFR by sterical hindrance or by the presence (or absence) of a specific tyrosine phosphorylation induced (or not induced) by ErbB2. It is also possible that a specific down-stream signal needed for the formation of or recruitment of the EGFR into clathrin coats at the plasma membrane is not induced from EGFR/ErbB2 heterodimers.

## 5.2 GA-INDUCED DOWNREGULATION OF ERBB2

In order to elucidate the mechanisms behind the endocytosis resistance of ErbB2, GA has been an important tool to increase the rate of ErbB2 downregulation. When Hsp70 and CHIP bind to the GA-induced ADP-bound like state of Hsp90 (see Figure 3), ErbB2 is ubiquitinated and subsequently internalized and degraded. Whether Hsp90 dissociates from this complex after GA binding is unclear. It is reported that GA induces dissociation of Hsp90 (Xu et al. 2001; Xu et al. 2002) although this does not fit with the existing models of Hsp90 cycling (Isaacs et al. 2003; Powers et al. 2007). There are also contradictory reports on where in the cell GA predominantly affects trafficking of ErbB2. These contradicting results are based on the understanding of ErbB2 trafficking, whether it is endocytosis resistant or whether it rapidly recycles, and thus whether GA induces endocytosis (Lerdrup et al. 2006; Lerdrup et al. 2007) or increased endosomal sorting for degradation (Austin et al. 2004). Based on confocal studies of ErbB2 localization in cells treated with monensin, it has previously been concluded that ErbB2 is not rapidly recycled but is endocytosis resistant (Longva et al. 2005). In Paper I, we also conclude that ErbB2 is endocytosis resistant. Therefore, the GA-induced downregulation of ErbB2 shown in Paper III must be a GA-induced endocytosis of ErbB2 rather than an inhibited recycling.

### 5.2.1 GA-INDUCED ENDOCYTOSIS OF ERBB2 IN HOMO- VERSUS HETERODIMERS

When studying the effect of GA on endocytosis of ErbB2, we found that GA induced downregulation of ErbB2 in cells expressing ErbB2 only (Paper III). We also found that the rate of downregulation was increased in cells additionally expressing EGFR and/or ErbB3 (Paper III). Why expression of EGFR or ErbB3 causes increased GA-induced downregulation of ErbB2 is not known, but the following two models could be proposed:

A) *GA-treatment is inefficient in cells containing ErbB2 only.* A low efficiency of GA on ErbB2 homodimers/-oligomers may have different explanations. The Hsp90-complex in a homodimer/-oligomer might be less available to GA because the ErbB2-Hsp90 association might be tighter or Hsp90 may be shielded between two ErbB2 molecules. In line with this idea, Hsp90 molecules exist in dimers (Richter et al. 2001; Pearl et al. 2006; Powers et al. 2006), and may bind two ErbB2 molecules simultaneously and thus stabilize ErbB2 homodimers more efficiently than heterodimers. We tested this possibility by measuring the initial effect of GA on ErbB2 homooligomers versus ErbB2 heterooligomers, but found that they appeared to be dissolved with similar kinetics.

B) *GA-treatment is similarly efficient in cells expressing only ErbB2 and in cells expressing an additional ErbB protein, but the action of GA is not sufficient for internalization of ErbB2*. A heterodimerization partner may recruit an accessory protein needed for efficient internalization of ErbB2 after GA-treatment.

Although the presence of EGFR or ErbB3 facilitates GA-induced downregulation of ErbB2, neither of these two receptors follows ErbB2 in GA-induced internalization (Paper III), suggesting that the dimers are dissolved before internalization.

## 5.3 THE ROLE OF UBIQUITIN IN ERBB PROTEIN ENDOCYTOSIS

Whether the GA-induced endocytosis of ErbB2 is a result of CHIP-mediated ubiquitination (Mimnaugh et al. 1996; Xu et al. 2002), or whether there are other mechanisms involved in its endocytosis is not clear. We investigated phosphorylation, but found that GA had no effect on the phosphorylation status of the ErbB proteins. There are at present no reports on GA-induced recruitment of additional proteins that may be candidates for induction of endocytosis. In agreement with previous findings (Mimnaugh et al. 1996; Xu et al. 2002), we found that ErbB2 was ubiquitinated upon treatment with GA (unpublished results). The correlation between ubiquitination and endocytosis is thus observed both for GA-induced endocytosis of ErbB2 and for ligand-induced endocytosis of EGFR. This might indicate that ubiquitination, as consequence of GA-treatment, can induce endocytosis of ErbB2.

For EGFR, the role of ubiquitination in endocytosis has been studied in more detail, and although there are no absolute proofs that ubiquitination of EGFR is required for its endocytosis, a sum of correlations points in that direction. There are several reports that reduced ubiquitination inhibits EGFR endocytosis. Examples are overexpression of Cbl mutants (Thien et al. 2001) and the Cbl-binding proteins TULA (Feshchenko et al. 2004; Kowanetz et al. 2004) and Sprouty (Wong et al. 2002; Fong et al. 2003; Stang et al. 2004) (see section 1.5.3). It was suggested that overexpression of TULA specifically inhibited endocytosis of EGFR by sequestering Cbl. In Paper IV, however, we demonstrated that overexpression of TULA inhibited several forms of endocytosis, including clathrin independent pathways, by sequestering dynamin. However, endocytosis of all proteins found to be inhibited by TULA overexpression, except the EGFR, were rescued upon overexpression of dynamin. This shows that Cbl, and thus probably ubiquitination, is required for EGFR endocytosis.

Cbl has been suggested to play roles in regulation of EGFR which are independent of the Cbl ubiquitin ligase activity. Phosphorylated Cbl is suggested to mediate recruitment of the CIN85-endophilin complex to the activated EGFR and thereby to regulate EGFR endocytosis (Soubeyran et al 2002) through endophilin's possible ability to induce plasma membrane curvature (reviewed in Reutens et al. 2002). There are,

however, several arguments against the importance of this role of Cbl in endocytosis. First, overexpression of dominant negative CIN85, or Cbl with mutated CIN85 binding sites, does not have a specific or essential effect on endocytosis of the EGFR (Jiang et al. 2003b). Second, EGFR internalization mediated by the Cbl RING finger domain, constituting the ubiquitin ligase activity, is four times more effective than internalization mediated by the Cbl C-terminal domain, interacting with the CIN85-endophilin complex (Huang et al. 2005). Third, the activity of endophilin mediating increased membrane curvature has been suggested to be a result of an experimental artefact (Gallop et al. 2005). The effects we have seen upon sequestering of Cbl (Paper IV) are therefore likely correlating with the ubiquitin ligase activity of Cbl.

## 5.4 ENDOCYTOSIS OF ERBB3

### 5.4.1 ERBB3, ENDOCYTOSIS IMPAIRED OR CONSTITUTIVELY ENDOCYTOSED?

The internalization of heregulin in cells expressing ErbB3 is slow compared to internalization of ligands that bind to the EGFR, and it has therefore been concluded that ErbB3 is endocytosis impaired (Baulida et al. 1996; Baulida et al. 1997 ; Waterman et al. 1998). There are also reports concluding that heregulin does not significantly affect downregulation of ErbB3 (Baulida et al. 1997; Qiu et al. 2002). The finding that ErbB3 is constitutively endocytosed (Paper II) is not contradictory to these reports, but rather explains why the internalization rate of heregulin is ‘slow’ compared to EGF; ligand-induced endocytosis is more rapid and efficient than constitutive endocytosis. The constitutive endocytosis observed in Paper II may thus also explain why the level of ErbB3 is not affected by exposure to ligand. In our study we used cells expressing both EGFR and ErbB3 and found a constitutive heterodimerization between ErbB3 and EGFR. It can be argued that a possible low level of constitutive EGFR-induced activation of ErbB3 might induce the observed constitutive endocytosis of ErbB3, and that the endocytosis is an artefact due to the high expression levels of the receptors. However, as many cancer cells overexpress both receptors (Citri et al. 2003), the effect overexpressed receptors may have on each other is clearly biologically relevant.

### 5.4.2 EGFR ENDOCYTOSIS IS NOT AFFECTED BY ERBB3

We found that overexpression of ErbB3, in contrast to overexpression of ErbB2, did not affect ligand induced endocytosis of EGFR. We can, however, not exclude that an inhibition of EGFR endocytosis could occur if the expression level of ErbB3 at the plasma membrane had been higher. Indeed, the level of ErbB2 at the plasma membrane in the PAE.EGFR.ErbB2 cells is significantly higher than the level of ErbB3 in the PAE.EGFR.ErbB3 cells. However, when selecting clones of cells transfected with ErbB3, we could not find cells with ErbB3 expression levels as high as found for ErbB2. As the half-life of ErbB3 is relatively short and as ErbB3 apparently readily enters clathrin-coated pits for internalization, it may not be expected to restrict the endocytosis of EGFR, even if expressed at high levels at the plasma membrane.

## 5.5 CONCLUSIONS AND PERSPECTIVES

### 5.5.1 ENDOCYTOSIS OF ERBB2

We conclude that ErbB2 is endocytosis resistant and that EGFR/ErbB2 heterodimers are not able to induce clathrin-coated pits upon incubation with EGF (Paper I). Since it is not known in detail what mediates localization of the EGFR to clathrin-coated pits, it is difficult to predict the reason for the ErbB2 induced inhibition. However, by investigating interaction partners of EGFR homodimers versus proteins interacting with heterodimers of ErbB2, possible differences might be found. Such studies are currently going on in our group. We also conclud that although GA can induce endocytosis of ErbB2 in cells expressing ErbB2 only, the presence of EGFR or ErbB3 makes the downregulation more efficient. Why the presence of EGFR and/or ErbB3 affects GA-induced endocytosis of ErbB2 is still unclear, and also in this case further studies of interaction partners of the different ErbB proteins are of high priority.

### 5.5.2 ENDOCYTOSIS OF ERBB3

We conclude that ErbB3 is constitutively localized both to the plasma membrane and to EEA1-positive endosomes. We further conclude that this is because ErbB3 is constitutively endocytosed in a clathrin-dependent manner. A further investigation of

this endocytosis is currently going on. This includes investigating whether the clathrin mediated endocytosis of ErbB3 depends on some of the same adaptors as EGFR, such as epsin and AP2. An additional important issue will be to compare the obtained results with results in cells expressing ErbB3 only. Such studies will elucidate the possible role of EGFR as heterodimerization partner and provide an answer to whether a constitutive association with, and phosphorylation induced by, EGFR is of importance. Because of the high constant level of intracellularly localized ErbB3, one could speculate that ErbB3 may play a more significant role intracellularly than EGFR and ErbB2 do. Intracellular signaling is shown to occur for EGFR (Wang et al. 2002), and the duration of MAPK signaling is reported to vary according to how EGFR is sorted following endocytosis (Malerod et al. 2007). Although ErbB3 is kinase deficient, it contains several phosphorylation sites that recruit e.g. the p85 subunit of PI3K (see Figure 5) after transphosphorylation. PI3K may potentially sustain intracellular signaling if ErbB3 is subsequently endocytosed in the absence of the heterodimerization partner. The importance of the intracellularly localized ErbB3 and the possible factors regulating ErbB3 localization and internalization, both on the plasma membrane and on endosomes, are of great interest for future studies.

### 5.5.3 UBIQUITINATION AND ENDOCYTOSIS

We conclude that overexpression of TULA inhibits dynamin dependent endocytosis. This finding has implications for further studies using TULA as a tool to mediate sequestering of Cbl, but may also be of interest for studies of the endogenous role of TULA in T-cells. We further conclude that the binding of TULA to dynamin is not the only reason for the inhibition of EGFR endocytosis. The inhibition of EGFR endocytosis is also caused by TULA binding to Cbl, and reveals a correlation between inhibition of ubiquitination and inhibition of EGFR endocytosis. Further investigation on the role of ubiquitination in endocytosis of the EGFR is currently going on in our group and includes mutation of ubiquitination sites in EGFR and chemical inhibition of ubiquitination. Unpublished data obtained in our group demonstrate that epsin mediates recruitment of ubiquitinated EGFR into clathrin-coated pits at the plasma membrane. As ubiquitination may be of importance in GA-induced endocytosis of ErbB2, studying a possible role of epsin is therefore interesting also in the endocytosis of this receptor.

The role of GA-induced ubiquitination can further be investigated by RNAi-mediated knock-down of CHIP or by inhibiting ubiquitination chemically. Also for ErbB3 it is reported that ubiquitination, mediated by Nrdp1, affects its downregulation (Diamonti et al. 2002; Qiu et al. 2002). It also is possible that ubiquitination of ErbB3 may correlate with endocytosis, and investigating a possible role of epsin also in endocytosis of ErbB3 is therefore of interest.

# REFERENCES


Abd El-Rehim, D. M., S. E. Pinder, C. E. Paish, J. A. Bell, R. S. Rampaul, R. W. Blamey, J. F. Robertson, R. I. Nicholson, and I. O. Ellis. 2004. Expression and co-expression of the members of the epidermal growth factor receptor (EGFR) family in invasive breast carcinoma. Br J Cancer **91**:1532-1542.

Alberts, B. J., Alexander; Lewis, Julian; Raff, Martin; Roberts, Keith; Walter, Peter. 2002. Molecular biology of the cell. Garland Science, Taylor & Francis Group.

Alimandi, M., L. M. Wang, D. Bottaro, C. C. Lee, A. Kuo, M. Frankel, P. Fedi, C. Tang, M. Lippman, and J. H. Pierce. 1997. Epidermal growth factor and betacellulin mediate signal transduction through co-expressed ErbB2 and ErbB3 receptors. Embo J **16**:5608-5617.

Alwan, H. A., E. J. van Zoelen, and J. E. van Leeuwen. 2003. Ligand-induced lysosomal epidermal growth factor receptor (EGFR) degradation is preceded by proteasome-dependent EGFR de-ubiquitination. J Biol Chem **278**:35781-35790.

Aqeilan, R. I., V. Donati, A. Palamarchuk, F. Trapasso, M. Kaou, Y. Pekarsky, M. Sudol, and C. M. Croce. 2005. WW domain-containing proteins, WWOX and YAP, compete for interaction with ErbB-4 and modulate its transcriptional function. Cancer Res **65**:6764-6772.

Arora, A., and E. M. Scholar. 2005. Role of tyrosine kinase inhibitors in cancer therapy. J Pharmacol Exp Ther **315**:971-979.

Asao, H., Y. Sasaki, T. Arita, N. Tanaka, K. Endo, H. Kasai, T. Takeshita, Y. Endo, T. Fujita, and K. Sugamura. 1997. Hrs is associated with STAM, a signal-transducing adaptor molecule. Its suppressive effect on cytokine-induced cell growth. J Biol Chem **272**:32785-32791.

Austin, C. D., A. M. De Maziere, P. I. Pisacane, S. M. van Dijk, C. Eigenbrot, M. X. Sliwkowski, J. Klumperman, and R. H. Scheller. 2004. Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. Mol Biol Cell **15**:5268-5282.

Authier, F., M. Metioui, A. W. Bell, and J. S. Mort. 1999. Negative regulation of epidermal growth factor signaling by selective proteolytic mechanisms in the endosome mediated by cathepsin B. J Biol Chem **274**:33723-33731.

Babst, M., D. J. Katzmann, E. J. Estepa-Sabal, T. Meerloo, and S. D. Emr. 2002a. Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting. Dev Cell **3**:271-282.

Babst, M., D. J. Katzmann, W. B. Snyder, B. Wendland, and S. D. Emr. 2002b. Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body. Dev Cell **3**:283-289.

Barnes, N. L., S. Khavari, G. P. Boland, A. Cramer, W. F. Knox, and N. J. Bundred. 2005. Absence of HER4 expression predicts recurrence of ductal carcinoma in situ of the breast. Clin Cancer Res **11**:2163-2168.

Barriere, H., C. Nemes, D. Lechardeur, M. Khan-Mohammad, K. Fruh, and G. L. Lukacs. 2006. Molecular basis of oligoubiquitin-dependent internalization of membrane proteins in Mammalian cells. Traffic **7**:282-297.

Baselga, J. 2001. The EGFR as a target for anticancer therapy--focus on cetuximab. Eur J Cancer **37 Suppl 4**:S16-22.

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

Baulida, J., and G. Carpenter. 1997. Heregulin degradation in the absence of rapid receptor-mediated internalization. Exp Cell Res **232**:167-172.

Baulida, J., M. H. Kraus, M. Alimandi, P. P. Di Fiore, and G. Carpenter. 1996. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. J Biol Chem **271**:5251-5257.

Bean, A. J., S. Davanger, M. F. Chou, B. Gerhardt, S. Tsujimoto, and Y. Chang. 2000. Hrs-2 regulates receptor-mediated endocytosis via interactions with Eps15. J Biol Chem **275**:15271-15278.

Beattie, E. C., R. C. Carroll, X. Yu, W. Morishita, H. Yasuda, M. von Zastrow, and R. C. Malenka. 2000. Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. Nat Neurosci **3**:1291-1300.

Benmerah, A., M. Bayrou, N. Cerf-Bensussan, and A. Dautry-Varsat. 1999. Inhibition of clathrin-coated pit assembly by an Eps15 mutant. J Cell Sci **112 ( Pt 9)**:1303-1311.

Benmerah, A., V. Poupon, N. Cerf-Bensussan, and A. Dautry-Varsat. 2000. Mapping of Eps15 domains involved in its targeting to clathrin-coated pits. J Biol Chem **275**:3288-3295.

Berger, M. B., J. M. Mendrola, and M. A. Lemmon. 2004. ErbB3/HER3 does not homodimerize upon neuregulin binding at the cell surface. FEBS Lett **569**:332-336.

Bieche, I., P. Onody, S. Tozlu, K. Driouch, M. Vidaud, and R. Lidereau. 2003. Prognostic value of ERBB family mRNA expression in breast carcinomas. Int J Cancer **106**:758-765.

Birrane, G., J. Chung, and J. A. Ladias. 2003. Novel mode of ligand recognition by the Erbin PDZ domain. J Biol Chem **278**:1399-1402.

Biscardi, J. S., M. C. Maa, D. A. Tice, M. E. Cox, T. H. Leu, and S. J. Parsons. 1999. c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. J Biol Chem **274**:8335-8343.

Bonifacino, J. S., and L. M. Traub. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem **72**:395-447.

Borg, J. P., S. Marchetto, A. Le Bivic, V. Ollendorff, F. Jaulin-Bastard, H. Saito, E. Fournier, J. Adelaide, B. Margolis, and D. Birnbaum. 2000. ERBIN: a basolateral PDZ protein that interacts with the mammalian ERBB2/HER2 receptor. Nat Cell Biol **2**:407-414.

Bouyain, S., and D. J. Leahy. 2007. Structure-based mutagenesis of the substrate-recognition domain of Nrdp1/FLRF identifies the binding site for the receptor tyrosine kinase ErbB3. Protein Sci **16**:654-661.

Bouyain, S., P. A. Longo, S. Li, K. M. Ferguson, and D. J. Leahy. 2005. The extracellular region of ErbB4 adopts a tethered conformation in the absence of ligand. Proc Natl Acad Sci U S A **102**:15024-15029.

Brown, D. A., and E. London. 1998. Structure and origin of ordered lipid domains in biological membranes. J Membr Biol **164**:103-114.

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

Burgess, A. W., H. S. Cho, C. Eigenbrot, K. M. Ferguson, T. P. Garrett, D. J. Leahy, M. A. Lemmon, M. X. Sliwkowski, C. W. Ward, and S. Yokoyama. 2003. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. Mol Cell **12**:541-552.

Cao, Z., X. Wu, L. Yen, C. Sweeney, and K. L. Carraway, 3rd. 2007. Neuregulin-Induced ErbB3 Downregulation is Mediated by a, Protein Stability Cascade Involving the E3 Ubiquitin Ligase Nrdp1. Mol Cell Biol.

Carpenter, G. 2000. The EGF receptor: a nexus for trafficking and signaling. Bioessays **22**:697-707.

Carpenter, G., L. King, Jr., and S. Cohen. 1978. Epidermal growth factor stimulates phosphorylation in membrane preparations in vitro. Nature **276**:409-410.

Carpentier, J. L., P. Gorden, R. G. Anderson, J. L. Goldstein, M. S. Brown, S. Cohen, and L. Orci. 1982. Co-localization of 125I-epidermal growth factor and ferritin-low density lipoprotein in coated pits: a quantitative electron microscopic study in normal and mutant human fibroblasts. J Cell Biol **95**:73-77.

Chen, H., S. Fre, V. I. Slepnev, M. R. Capua, K. Takei, M. H. Butler, P. P. Di Fiore, and P. De Camilli. 1998. Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. Nature **394**:793-797.

Chen, X., G. Levkowitz, E. Tzahar, D. Karunagaran, S. Lavi, N. Ben-Baruch, O. Leitner, B. J. Ratzkin, S. S. Bacus, and Y. Yarden. 1996. An immunological approach reveals biological differences between the two NDF/heregulin receptors, ErbB-3 and ErbB-4. J Biol Chem **271**:7620-7629.

Cho, H. S., and D. J. Leahy. 2002. Structure of the extracellular region of HER3 reveals an interdomain tether. Science **297**:1330-1333.

Citri, A., I. Alroy, S. Lavi, C. Rubin, W. Xu, N. Grammatikakis, C. Patterson, L. Neckers, D. W. Fry, and Y. Yarden. 2002. Drug-induced ubiquitylation and degradation of ErbB receptor tyrosine kinases: implications for cancer therapy. Embo J **21**:2407-2417.

Citri, A., J. Gan, Y. Mosesson, G. Vereb, J. Szollosi, and Y. Yarden. 2004. Hsp90 restrains ErbB-2/HER2 signalling by limiting heterodimer formation. EMBO Rep **5**:1165-1170.

Citri, A., K. B. Skaria, and Y. Yarden. 2003. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. Exp Cell Res **284**:54-65.

Cohen, S. 1962. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J Biol Chem **237**:1555-1562.

Cohen, S. 1964. Isolation and Biological Effects of an Epidermal Growth-Stimulating Protein. Natl Cancer Inst Monogr **13**:13-37.

Conner, S. D., and S. L. Schmid. 2003a. Regulated portals of entry into the cell. Nature **422**:37-44.

Conner, S. D., and S. L. Schmid. 2003b. Differential requirements for AP-2 in clathrin-mediated endocytosis. J Cell Biol **162**:773-779.

Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. Proc Natl Acad Sci U S A **80**:2258-2262.

Dawson, J. P., Z. Bu, and M. A. Lemmon. 2007. Ligand-induced structural transitions in ErbB receptor extracellular domains. Structure **15**:942-954.

De Camilli, P., and K. Takei. 1996. Molecular mechanisms in synaptic vesicle endocytosis and recycling. Neuron **16**:481-486.

deFazio, A., Y. E. Chiew, R. L. Sini, P. W. Janes, and R. L. Sutherland. 2000. Expression of c-erbB receptors, heregulin and oestrogen receptor in human breast cell lines. Int J Cancer **87**:487-498.

Di Fiore, P. P., and P. De Camilli. 2001. Endocytosis and signaling. an inseparable partnership. Cell **106**:1-4.

Di Paolo, G., and P. De Camilli. 2006. Phosphoinositides in cell regulation and membrane dynamics. Nature **443**:651-657.

Diamonti, A. J., P. M. Guy, C. Ivanof, K. Wong, C. Sweeney, and K. L. Carraway, 3rd. 2002. An RBCC protein implicated in maintenance of steady-state neuregulin receptor levels. Proc Natl Acad Sci U S A **99**:2866-2871.

Duan, L., Y. Miura, M. Dimri, B. Majumder, I. L. Dodge, A. L. Reddi, A. Ghosh, N. Fernandes, P. Zhou, K. Mullane-Robinson, N. Rao, S. Donoghue, R. A. Rogers, D. Bowtell, M. Naramura, H. Gu, V. Band, and H. Band. 2003. Cbl-mediated ubiquitinylation is required for lysosomal sorting of epidermal growth factor receptor but is dispensable for endocytosis. J Biol Chem **278**:28950-28960.

Duncan, L. M., S. Piper, R. B. Dodd, M. K. Saville, C. M. Sanderson, J. P. Luzio, and P. J. Lehner. 2006. Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. Embo J **25**:1635-1645.

Ebner, R., and R. Derynck. 1991. Epidermal growth factor and transforming growth factor-alpha: differential intracellular routing and processing of ligand-receptor complexes. Cell Regul **2**:599-612.

Edeling, M. A., C. Smith, and D. Owen. 2006. Life of a clathrin coat: insights from clathrin and AP structures. Nat Rev Mol Cell Biol **7**:32-44.

Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411**:494-498.

Elenius, K., C. J. Choi, S. Paul, E. Santiestevan, E. Nishi, and M. Klagsbrun. 1999. Characterization of a naturally occurring ErbB4 isoform that does not bind or activate phosphatidyl inositol 3-kinase. Oncogene **18**:2607-2615.

Elenius, K., G. Corfas, S. Paul, C. J. Choi, C. Rio, G. D. Plowman, and M. Klagsbrun. 1997. A novel juxtamembrane domain isoform of HER4/ErbB4. Isoform-specific tissue distribution and differential processing in response to phorbol ester. J Biol Chem **272**:26761-26768.

Fang, D., H. Y. Wang, N. Fang, Y. Altman, C. Elly, and Y. C. Liu. 2001. Cbl-b, a RING-type E3 ubiquitin ligase, targets phosphatidylinositol 3-kinase for ubiquitination in T cells. J Biol Chem **276**:4872-4878.

Ferguson, K. M., M. B. Berger, J. M. Mendrola, H. S. Cho, D. J. Leahy, and M. A. Lemmon. 2003. EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Mol Cell **11**:507-517.

Feshchenko, E. A., E. V. Smirnova, G. Swaminathan, A. M. Teckchandani, R. Agrawal, H. Band, X. Zhang, R. S. Annan, S. A. Carr, and A. Y. Tsygankov. 2004. TULA: an SH3- and UBA-containing protein that binds to c-Cbl and ubiquitin. Oncogene **23**:4690-4706.

Fong, C. W., H. F. Leong, E. S. Wong, J. Lim, P. Yusoff, and G. R. Guy. 2003. Tyrosine phosphorylation of Sprouty2 enhances its interaction with c-Cbl and is crucial for its function. J Biol Chem **278**:33456-33464.

Ford, M. G., I. G. Mills, B. J. Peter, Y. Vallis, G. J. Praefcke, P. R. Evans, and H. T. McMahon. 2002. Curvature of clathrin-coated pits driven by epsin. Nature **419**:361-366.

Frederick, L., X. Y. Wang, G. Eley, and C. D. James. 2000. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. Cancer Res **60**:1383-1387.

French, A. R., D. K. Tadaki, S. K. Niyogi, and D. A. Lauffenburger. 1995. Intracellular trafficking of epidermal growth factor family ligands is directly influenced by the pH sensitivity of the receptor/ligand interaction. J Biol Chem **270**:4334-4340.

Futter, C. E., A. Pearse, L. J. Hewlett, and C. R. Hopkins. 1996. Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. J Cell Biol **132**:1011-1023.

Gallop, J. L., P. J. Butler, and H. T. McMahon. 2005. Endophilin and CtBP/BARS are not acyl transferases in endocytosis or Golgi fission. Nature **438**:675-678.

Garrett, T. P., N. M. McKern, M. Lou, T. C. Elleman, T. E. Adams, G. O. Lovrecz, M. Kofler, R. N. Jorissen, E. C. Nice, A. W. Burgess, and C. W. Ward. 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.

Gilbertson, R., R. Hernan, T. Pietsch, L. Pinto, P. Scotting, R. Allibone, D. Ellison, R. Perry, A. Pearson, and J. Lunec. 2001. Novel ERBB4 juxtamembrane splice variants are frequently expressed in childhood medulloblastoma. Genes Chromosomes Cancer **31**:288-294.

Gilbertson, R. J., R. H. Perry, P. J. Kelly, A. D. Pearson, and J. Lunec. 1997. Prognostic significance of HER2 and HER4 coexpression in childhood medulloblastoma. Cancer Res **57**:3272-3280.

Goldstein, N. I., M. Prewett, K. Zuklys, P. Rockwell, and J. Mendelsohn. 1995. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. Clin Cancer Res **1**:1311-1318.

Grandal, M. V., R. Zandi, M. W. Pedersen, B. M. Willumsen, B. van Deurs, and H. S. Poulsen. 2007. EGFRvIII escapes down-regulation due to impaired internalization and sorting to lysosomes. Carcinogenesis **28**:1408-1417.

Grovdal, L. M., E. Stang, A. Sorkin, and I. H. Madshus. 2004. Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation. Exp Cell Res **300**:388-395.

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

Haglund, K., S. Sigismund, S. Polo, I. Szymkiewicz, P. P. Di Fiore, and I. Dikic. 2003. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol **5**:461-466.

Hallberg, B., S. I. Rayter, and J. Downward. 1994. Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. J Biol Chem **269**:3913-3916.

Hamilton, A. J., and D. C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286**:950-952.

Han, W., T. Zhang, H. Yu, J. G. Foulke, and C. K. Tang. 2006. Hypophosphorylation of residue Y1045 leads to defective downregulation of EGFRvIII. Cancer Biol Ther **5**:1361-1368.

Hanover, J. A., M. C. Willingham, and I. Pastan. 1984. Kinetics of transit of transferrin and epidermal growth factor through clathrin-coated membranes. Cell **39**:283-293.

Hartley, D., H. Meisner, and S. Corvera. 1995. Specific association of the beta isoform of the p85 subunit of phosphatidylinositol-3 kinase with the proto-oncogene c-cbl. J Biol Chem **270**:18260-18263.

Hawryluk, M. J., P. A. Keyel, S. K. Mishra, S. C. Watkins, J. E. Heuser, and L. M. Traub. 2006. Epsin 1 is a polyubiquitin-selective clathrin-associated sorting protein. Traffic **7**:262-281.

Heilker, R., M. Spiess, and P. Crottet. 1999. Recognition of sorting signals by clathrin adaptors. Bioessays **21**:558-567.

Hendriks, B. S., L. K. Opresko, H. S. Wiley, and D. Lauffenburger. 2003. Coregulation of epidermal growth factor receptor/human epidermal growth factor receptor 2 (HER2) levels and locations: quantitative analysis of HER2 overexpression effects. Cancer Res **63**:1130-1137.

Henley, J. R., E. W. Krueger, B. J. Oswald, and M. A. McNiven. 1998. Dynamin-mediated internalization of caveolae. J Cell Biol **141**:85-99.

Hinshaw, J. E. 2000. Dynamin and its role in membrane fission. Annu Rev Cell Dev Biol **16**:483-519.

Holbro, T., R. R. Beerli, F. Maurer, M. Koziczak, C. F. Barbas, 3rd, and N. E. Hynes. 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**:8933-8938.

Holbro, T., and N. E. Hynes. 2004. ErbB receptors: directing key signaling networks throughout life. Annu Rev Pharmacol Toxicol **44**:195-217.

Hommelgaard, A. M., M. Lerdrup, and B. van Deurs. 2004. Association with membrane protrusions makes ErbB2 an internalization-resistant receptor. Mol Biol Cell **15**:1557-1567.

Hommelgaard, A. M., K. Roepstorff, F. Vilhardt, M. L. Torgersen, K. Sandvig, and B. van Deurs. 2005. Caveolae: stable membrane domains with a potential for internalization. Traffic **6**:720-724.

Huang, F., L. K. Goh, and A. Sorkin. 2007. EGF receptor ubiquitination is not necessary for its internalization. Proc Natl Acad Sci U S A **104**:16904-16909.

Huang, F., X. Jiang, and A. Sorkin. 2003. Tyrosine phosphorylation of the beta2 subunit of clathrin adaptor complex AP-2 reveals the role of a di-leucine motif in the epidermal growth factor receptor trafficking. J Biol Chem **278**:43411-43417.

Huang, F., A. Khvorova, W. Marshall, and A. Sorkin. 2004. Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. J Biol Chem **279**:16657-16661.

Huang, F., D. Kirkpatrick, X. Jiang, S. Gygi, and A. Sorkin. 2006. Differential Regulation of EGF Receptor Internalization and Degradation by Multiubiquitination within the Kinase Domain. Mol Cell **21**:737-748.

Huang, F., and A. Sorkin. 2005. Growth factor receptor binding protein 2-mediated recruitment of the RING domain of Cbl to the epidermal growth factor receptor is essential and sufficient to support receptor endocytosis. Mol Biol Cell **16**:1268-1281.

Hynes, N. E., and H. A. Lane. 2005. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer **5**:341-354.

Isaacs, J. S., W. Xu, and L. Neckers. 2003. Heat shock protein 90 as a molecular target for cancer therapeutics. Cancer Cell **3**:213-217.

Itoh, T., K. S. Erdmann, A. Roux, B. Habermann, H. Werner, and P. De Camilli. 2005. Dynamin and the actin cytoskeleton cooperatively regulate plasma membrane invagination by BAR and F-BAR proteins. Dev Cell **9**:791-804.

Jaulin-Bastard, F., H. Saito, A. Le Bivic, V. Ollendorff, S. Marchetto, D. Birnbaum, and J. P. Borg. 2001. The ERBB2/HER2 receptor differentially interacts with ERBIN and PICK1 PSD-95/DLG/ZO-1 domain proteins. J Biol Chem **276**:15256-15263.

Jiang, X., F. Huang, A. Marusyk, and A. Sorkin. 2003a. Grb2 regulates internalization of EGF receptors through clathrin-coated pits. Mol Biol Cell **14**:858-870.

Jiang, X., and A. Sorkin. 2003b. Epidermal growth factor receptor internalization through clathrin-coated pits requires Cbl RING finger and proline-rich domains but not receptor polyubiquitylation. Traffic **4**:529-543.

Johannes, L., and C. Lamaze. 2002. Clathrin-dependent or not: is it still the question? Traffic **3**:443-451.

Johannessen, L. E., N. M. Pedersen, K. W. Pedersen, I. H. Madshus, and E. Stang. 2006. Activation of the epidermal growth factor (EGF) receptor induces formation of EGF receptor- and Grb2-containing clathrin-coated pits. Mol Cell Biol **26**:389-401.

Jones, R. B., A. Gordus, J. A. Krall, and G. MacBeath. 2006. A quantitative protein interaction network for the ErbB receptors using protein microarrays. Nature **439**:168-174.

Jorissen, R. N., F. Walker, N. Pouliot, T. P. Garrett, C. W. Ward, and A. W. Burgess. 2003. Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res **284**:31-53.

Junttila, T. T., M. Laato, T. Vahlberg, K. O. Soderstrom, T. Visakorpi, J. Isola, and K. Elenius. 2003. Identification of patients with transitional cell carcinoma of the bladder overexpressing ErbB2, ErbB3, or specific ErbB4 isoforms: real-time reverse transcription-PCR analysis in estimation of ErbB receptor status from cancer patients. Clin Cancer Res **9**:5346-5357.

Kani, K., C. M. Warren, C. S. Kaddis, J. A. Loo, and R. Landgraf. 2005. Oligomers of ERBB3 have two distinct interfaces that differ in their sensitivity to disruption by heregulin. J Biol Chem **280**:8238-8247.

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.

Katzmann, D. J., M. Babst, and S. D. Emr. 2001. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell **106**:145-155.

Kazazic, M., K. Roepstorff, L. E. Johannessen, N. M. Pedersen, B. van Deurs, E. Stang, and I. H. Madshus. 2006. EGF-induced activation of the EGF receptor does not trigger mobilization of caveolae. Traffic **7**:1518-1527.

Kim, H. H., U. Vijapurkar, N. J. Hellyer, D. Bravo, and J. G. Koland. 1998. Signal transduction by epidermal growth factor and heregulin via the kinase-deficient ErbB3 protein. Biochem J **334 ( Pt 1)**:189-195.

Kirchhausen, T., J. S. Bonifacino, and H. Riezman. 1997. Linking cargo to vesicle formation: receptor tail interactions with coat proteins. Curr Opin Cell Biol **9**:488-495.

Kirkham, M., and R. G. Parton. 2005. Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. Biochim Biophys Acta **1745**:273-286.

Klapper, L. N., S. Glathe, N. Vaisman, N. E. Hynes, G. C. Andrews, M. Sela, and Y. Yarden. 1999. The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. Proc Natl Acad Sci U S A **96**:4995-5000.

Klein, P., D. Mattoon, M. A. Lemmon, and J. Schlessinger. 2004. A structure-based model for ligand binding and dimerization of EGF receptors. Proc Natl Acad Sci U S A **101**:929-934.

Kloth, M. T., K. K. Laughlin, J. S. Biscardi, J. L. Boerner, S. J. Parsons, and C. M. Silva. 2003. STAT5b, a Mediator of Synergism between c-Src and the Epidermal Growth Factor Receptor. J Biol Chem **278**:1671-1679.

Kolch, W. 2000. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J **351 Pt 2**:289-305.

Kowanetz, K., N. Crosetto, K. Haglund, M. H. Schmidt, C. H. Heldin, and I. Dikic. 2004. Suppressors of T-cell receptor signaling Sts-1 and Sts-2 bind to Cbl and inhibit endocytosis of receptor tyrosine kinases. J Biol Chem **279**:32786-32795.

Kurzchalia, T. V., and R. G. Parton. 1999. Membrane microdomains and caveolae. Curr Opin Cell Biol **11**:424-431.

Landgraf, R., and D. Eisenberg. 2000. Heregulin reverses the oligomerization of HER3. Biochemistry **39**:8503-8511.

Lemmon, M. A. 2003. Phosphoinositide recognition domains. Traffic **4**:201-213.

Lenferink, A. E., R. Pinkas-Kramarski, M. L. van de Poll, M. J. van Vugt, L. N. Klapper, E. Tzahar, H. Waterman, M. Sela, E. J. van Zoelen, and Y. Yarden. 1998. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. Embo J **17**:3385-3397.

Leonard, W. J. 2001. Role of Jak kinases and STATs in cytokine signal transduction. Int J Hematol **73**:271-277.

Lerdrup, M., S. Bruun, M. V. Grandal, K. Roepstorff, M. M. Kristensen, A. M. Hommelgaard, and B. van Deurs. 2007. Endocytic down-regulation of ErbB2 is stimulated by cleavage of its C-terminus. Mol Biol Cell **18**:3656-3666.

Lerdrup, M., A. M. Hommelgaard, M. Grandal, and B. van Deurs. 2006. Geldanamycin stimulates internalization of ErbB2 in a proteasome-dependent way. J Cell Sci **119**:85-95.

Levkowitz, G., L. N. Klapper, E. Tzahar, A. Freywald, M. Sela, and Y. Yarden. 1996. Coupling of the c-Cbl protooncogene product to ErbB-1/EGF-receptor but not to other ErbB proteins. Oncogene **12**:1117-1125.

Levkowitz, G., H. Waterman, S. A. Ettenberg, M. Katz, A. Y. Tsygankov, I. Alroy, S. Lavi, K. Iwai, Y. Reiss, A. Ciechanover, S. Lipkowitz, and Y. Yarden. 1999. Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol Cell **4**:1029-1040.

Levkowitz, G., H. Waterman, E. Zamir, Z. Kam, S. Oved, W. Y. Langdon, L. Beguinot, B. Geiger, and Y. Yarden. 1998. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev **12**:3663-3674.

Li, S., K. R. Schmitz, P. D. Jeffrey, J. J. Wiltzius, P. Kussie, and K. M. Ferguson. 2005. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell **7**:301-311.

Lichtenberg, D., F. M. Goni, and H. Heerklotz. 2005. Detergent-resistant membranes should not be identified with membrane rafts. Trends Biochem Sci **30**:430-436.

Lombardo, C. R., T. G. Consler, and D. B. Kassel. 1995. In vitro phosphorylation of the epidermal growth factor receptor autophosphorylation domain by c-src: identification of phosphorylation sites and c-src SH2 domain binding sites. Biochemistry **34**:16456-16466.

Longva, K. E., F. D. Blystad, E. Stang, A. M. Larsen, L. E. Johannessen, and I. H. Madshus. 2002. Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. J Cell Biol **156**:843-854.

Longva, K. E., N. M. Pedersen, C. Haslekas, E. Stang, and I. H. Madshus. 2005. Herceptin-induced inhibition of ErbB2 signaling involves reduced phosphorylation of Akt but not endocytic down-regulation of ErbB2. Int J Cancer **116**:359-367.

Malerod, L., S. Stuffers, A. Brech, and H. Stenmark. 2007. Vps22/EAP30 in ESCRT-II mediates endosomal sorting of growth factor and chemokine receptors destined for lysosomal degradation. Traffic **8**:1617-1629.

Mimnaugh, E. G., C. Chavany, and L. Neckers. 1996. Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. J Biol Chem **271**:22796-22801.

Mineo, C., G. N. Gill, and R. G. Anderson. 1999. Regulated migration of epidermal growth factor receptor from caveolae. J Biol Chem **274**:30636-30643.

Miyake, S., M. L. Lupher, Jr., B. Druker, and H. Band. 1998. The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha. Proc Natl Acad Sci U S A **95**:7927-7932.

Mizuno, E., T. Iura, A. Mukai, T. Yoshimori, N. Kitamura, and M. Komada. 2005. Regulation of epidermal growth factor receptor down-regulation by UBPY-mediated deubiquitination at endosomes. Mol Biol Cell **16**:5163-5174.

Mosesson, Y., K. Shtiegman, M. Katz, Y. Zwang, G. Vereb, J. Szollosi, and Y. Yarden. 2003. Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. J Biol Chem **278**:21323-21326.

Motley, A., N. A. Bright, M. N. Seaman, and M. S. Robinson. 2003. Clathrin-mediated endocytosis in AP-2-depleted cells. J Cell Biol **162**:909-918.

Murata, S., Y. Minami, M. Minami, T. Chiba, and K. Tanaka. 2001. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep **2**:1133-1138.

Muthuswamy, S. K., M. Gilman, and J. S. Brugge. 1999. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. Mol Cell Biol **19**:6845-6857.

Naidu, R., M. Yadav, S. Nair, and M. K. Kutty. 1998. Expression of c-erbB3 protein in primary breast carcinomas. Br J Cancer **78**:1385-1390.

Narita, T., T. Taga, K. Sugita, S. Nakazawa, and S. Ohta. 2001. The autocrine loop of epidermal growth factor receptor-epidermal growth factor / transforming growth factor-alpha in malignant rhabdoid tumor cell lines: heterogeneity of autocrine mechanism in TTC549. Jpn J Cancer Res **92**:269-278.

Neckers, L. 2002. Hsp90 inhibitors as novel cancer chemotherapeutic agents. Trends Mol Med **8**:S55-61.

Nesterov, A., R. C. Kurten, and G. N. Gill. 1995a. Association of epidermal growth factor receptors with coated pit adaptins via a tyrosine phosphorylation-regulated mechanism. J Biol Chem **270**:6320-6327.

Nesterov, A., H. S. Wiley, and G. N. Gill. 1995b. Ligand-induced endocytosis of epidermal growth factor receptors that are defective in binding adaptor proteins. Proc Natl Acad Sci U S A **92**:8719-8723.

Ni, C. Y., M. P. Murphy, T. E. Golde, and G. Carpenter. 2001. gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science **294**:2179-2181.

Nicholson, R. I., J. M. Gee, and M. E. Harper. 2001. EGFR and cancer prognosis. Eur J Cancer **37 Suppl 4**:S9-15.

Nielsen, U. B., M. H. Cardone, A. J. Sinskey, G. MacBeath, and P. K. Sorger. 2003. Profiling receptor tyrosine kinase activation by using Ab microarrays. Proc Natl Acad Sci U S A **100**:9330-9335.

Nimnual, A., and D. Bar-Sagi. 2002. The two hats of SOS. Sci STKE **2002**:PE36.

Offterdinger, M., and P. I. Bastiaens. 2007. Prolonged EGFR Signaling by ERBB2-Mediated Sequestration at the Plasma Membrane. Traffic.

Ogiso, H., R. Ishitani, O. Nureki, S. Fukai, M. Yamanaka, J. H. Kim, K. Saito, A. Sakamoto, M. Inoue, M. Shirouzu, and S. Yokoyama. 2002. Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. Cell **110**:775-787.

Oh, P., D. P. McIntosh, and J. E. Schnitzer. 1998. Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. J Cell Biol **141**:101-114.

Olayioye, M. A., I. Beuvink, K. Horsch, J. M. Daly, and N. E. Hynes. 1999. ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. J Biol Chem **274**:17209-17218.

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

Omerovic, J., E. M. Puggioni, S. Napoletano, V. Visco, R. Fraioli, L. Frati, A. Gulino, and M. Alimandi. 2004. Ligand-regulated association of ErbB-4 to the transcriptional co-activator YAP65 controls transcription at the nuclear level. Exp Cell Res **294**:469-479.

Parton, R. G., B. Joggerst, and K. Simons. 1994. Regulated internalization of caveolae. J Cell Biol **127**:1199-1215.

Pearl, L. H., and C. Prodromou. 2006. Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu Rev Biochem **75**:271-294.

Pedersen, N. M., I. H. Madshus, C. Haslekås, and E. Stang. In press. Geldanamycin-induced Down-regulation of ErbB2 from the Plasma Membrane Is Clathrin-Dependent, but Independent of Proteasomal Activity. Molecular Cancer Research.

Pelkmans, L., and A. Helenius. 2002. Endocytosis via caveolae. Traffic **3**:311-320.

Pelkmans, L., J. Kartenbeck, and A. Helenius. 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat Cell Biol **3**:473-483.

Pelkmans, L., and M. Zerial. 2005. Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae. Nature **436**:128-133.

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

Polo, S., S. Sigismund, M. Faretta, M. Guidi, M. R. Capua, G. Bossi, H. Chen, P. De Camilli, and P. P. Di Fiore. 2002. A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature **416**:451-455.

Powers, M. V., and P. Workman. 2007. Inhibitors of the heat shock response: biology and pharmacology. FEBS Lett **581**:3758-3769.

Powers, M. V., and P. Workman. 2006. Targeting of multiple signalling pathways by heat shock protein 90 molecular chaperone inhibitors. Endocr Relat Cancer **13 Suppl 1**:S125-135.

Praefcke, G. J., M. G. Ford, E. M. Schmid, L. E. Olesen, J. L. Gallop, S. Y. Peak-Chew, Y. Vallis, M. M. Babu, I. G. Mills, and H. T. McMahon. 2004. Evolving nature of the AP2 alpha-appendage hub during clathrin-coated vesicle endocytosis. Embo J **23**:4371-4383.

Prenzel, N., O. M. Fischer, S. Streit, S. Hart, and A. Ullrich. 2001. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr Relat Cancer **8**:11-31.

Prewett, M. C., A. T. Hooper, R. Bassi, L. M. Ellis, H. W. Waksal, and D. J. Hicklin. 2002. Enhanced antitumor activity of anti-epidermal growth factor receptor monoclonal antibody IMC-C225 in combination with irinotecan (CPT-11) against human colorectal tumor xenografts. Clin Cancer Res **8**:994-1003.

Qiu, X. B., and A. L. Goldberg. 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**:14843-14848.

Raiborg, C., K. G. Bache, D. J. Gillooly, I. H. Madshus, E. Stang, and H. Stenmark. 2002a. Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat Cell Biol **4**:394-398.

Raiborg, C., B. Bremnes, A. Mehlum, D. J. Gillooly, A. D'Arrigo, E. Stang, and H. Stenmark. 2001. FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes. J Cell Sci **114**:2255-2263.

Raiborg, C., T. E. Rusten, and H. Stenmark. 2003. Protein sorting into multivesicular endosomes. Curr Opin Cell Biol **15**:446-455.

Raiborg, C., and H. Stenmark. 2002b. Hrs and endocytic sorting of ubiquitinated membrane proteins. Cell Struct Funct **27**:403-408.

Ram, T. G., H. L. Hosick, and S. P. Ethier. 2000. Heregulin-beta is especially potent in activating phosphatidylinositol 3-kinase in nontransformed human mammary epithelial cells. J Cell Physiol **183**:301-313.

Reutens, A. T., and C. G. Begley. 2002. Endophilin-1: a multifunctional protein. Int J Biochem Cell Biol **34**:1173-1177.

Richter, K., P. Muschler, O. Hainzl, and J. Buchner. 2001. Coordinated ATP hydrolysis by the Hsp90 dimer. J Biol Chem **276**:33689-33696.

Ringerike, T., F. D. Blystad, F. O. Levy, I. H. Madshus, and E. Stang. 2002. Cholesterol is important in control of EGF receptor kinase activity but EGF receptors are not concentrated in caveolae. J Cell Sci **115**:1331-1340.

Rio, C., J. D. Buxbaum, J. J. Peschon, and G. Corfas. 2000. Tumor necrosis factor-alpha-converting enzyme is required for cleavage of erbB4/HER4. J Biol Chem **275**:10379-10387.

Robinson, M. S. 2004. Adaptable adaptors for coated vesicles. Trends Cell Biol **14**:167-174.

Roskoski, R., Jr. 2004. The ErbB/HER receptor protein-tyrosine kinases and cancer. Biochem Biophys Res Commun **319**:1-11.

Ross, J. S., J. A. Fletcher, G. P. Linette, J. Stec, E. Clark, M. Ayers, W. F. Symmans, L. Pusztai, and K. J. Bloom. 2003. The Her-2/neu gene and protein in breast cancer 2003: biomarker and target of therapy. Oncologist **8**:307-325.

Sasaoka, T., W. J. Langlois, J. W. Leitner, B. Draznin, and J. M. Olefsky. 1994. The signaling pathway coupling epidermal growth factor receptors to activation of p21ras. J Biol Chem **269**:32621-32625.

Schlessinger, J. 2002. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell **110**:669-672.

Seto, E. S., H. J. Bellen, and T. E. Lloyd. 2002. When cell biology meets development: endocytic regulation of signaling pathways. Genes Dev **16**:1314-1336.

Sever, S., H. Damke, and S. L. Schmid. 2000. Garrotes, springs, ratchets, and whips: putting dynamin models to the test. Traffic **1**:385-392.

Sherrill, J. M., and J. Kyte. 1999. Kinetics of activation of the tyrosine kinase of a deletion mutant of epidermal growth factor receptor missing the sites of self-phosphorylation. Biochemistry **38**:3106-3111.

Shih, S. C., K. E. Sloper-Mould, and L. Hicke. 2000. Monoubiquitin carries a novel internalization signal that is appended to activated receptors. Embo J **19**:187-198.

Shoelson, S. E., M. Sivaraja, K. P. Williams, P. Hu, J. Schlessinger, and M. A. Weiss. 1993. Specific phosphopeptide binding regulates a conformational change in the PI 3-kinase SH2 domain associated with enzyme activation. Embo J **12**:795-802.

Sigismund, S., T. Woelk, C. Puri, E. Maspero, C. Tacchetti, P. Transidico, P. P. Di Fiore, and S. Polo. 2005. Clathrin-independent endocytosis of ubiquitinated cargos. Proc Natl Acad Sci U S A **102**:2760-2765.

Simonsen, A., A. E. Wurmser, S. D. Emr, and H. Stenmark. 2001. The role of phosphoinositides in membrane transport. Curr Opin Cell Biol **13**:485-492.

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

Smart, E. J., G. A. Graf, M. A. McNiven, W. C. Sessa, J. A. Engelman, P. E. Scherer, T. Okamoto, and M. P. Lisanti. 1999. Caveolins, liquid-ordered domains, and signal transduction. Mol Cell Biol **19**:7289-7304.

Sorkin, A., P. P. Di Fiore, and G. Carpenter. 1993. The carboxyl terminus of epidermal growth factor receptor/erbB-2 chimerae is internalization impaired. Oncogene **8**:3021-3028.

Sorkin, A., M. Mazzotti, T. Sorkina, L. Scotto, and L. Beguinot. 1996. Epidermal growth factor receptor interaction with clathrin adaptors is mediated by the Tyr974-containing internalization motif. J Biol Chem **271**:13377-13384.

Sorkin, A., and M. Von Zastrow. 2002. Signal transduction and endocytosis: close encounters of many kinds. Nat Rev Mol Cell Biol **3**:600-614.

Sorkina, T., F. Huang, L. Beguinot, and A. Sorkin. 2002. Effect of tyrosine kinase inhibitors on clathrin-coated pit recruitment and internalization of epidermal growth factor receptor. J Biol Chem **277**:27433-27441.

Stang, E., F. D. Blystad, M. Kazazic, V. Bertelsen, T. Brodahl, C. Raiborg, H. Stenmark, and I. H. Madshus. 2004. Cbl-dependent ubiquitination is required for progression of EGF receptors into clathrin-coated pits. Mol Biol Cell **15**:3591-3604.

Stang, E., L. E. Johannessen, S. L. Knardal, and I. H. Madshus. 2000. Polyubiquitination of the epidermal growth factor receptor occurs at the plasma membrane upon ligand-induced activation. J Biol Chem **275**:13940-13947.

Stenmark, H., R. Aasland, B. H. Toh, and A. D'Arrigo. 1996. Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. J Biol Chem **271**:24048-24054.

Stover, D. R., M. Becker, J. Liebetanz, and N. B. Lydon. 1995. Src phosphorylation of the epidermal growth factor receptor at novel sites mediates receptor interaction with Src and P85 alpha. J Biol Chem **270**:15591-15597.

Suo, Z., B. Risberg, M. G. Kalsson, K. Willman, A. Tierens, E. Skovlund, and J. M. Nesland. 2002. EGFR family expression in breast carcinomas. c-erbB-2 and c-erbB-4 receptors have different effects on survival. J Pathol **196**:17-25.

Sweeney, C., J. K. Miller, D. L. Shattuck, and K. L. Carraway, 3rd. 2006. ErbB receptor negative regulatory mechanisms: implications in cancer. J Mammary Gland Biol Neoplasia **11**:89-99.

Tagawa, A., A. Mezzacasa, A. Hayer, A. Longatti, L. Pelkmans, and A. Helenius. 2005. Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. J Cell Biol **170**:769-779.

Thien, C. B., F. Walker, and W. Y. Langdon. 2001. RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation. Mol Cell **7**:355-365.

Thomsen, P., K. Roepstorff, M. Stahlhut, and B. van Deurs. 2002. Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. Mol Biol Cell **13**:238-250.

Tikhomirov, O., and G. Carpenter. 2003. Identification of ErbB-2 kinase domain motifs required for geldanamycin-induced degradation. Cancer Res **63**:39-43.

Tikhomirov, O., and G. Carpenter. 2001. Caspase-dependent cleavage of ErbB-2 by geldanamycin and staurosporin. J Biol Chem **276**:33675-33680.

Tikhomirov, O., and G. Carpenter. 2000. Geldanamycin induces ErbB-2 degradation by proteolytic fragmentation. J Biol Chem **275**:26625-26631.

Traub, L. M. 2003. Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. J Cell Biol **163**:203-208.

Tzahar, E., H. Waterman, X. Chen, G. Levkowitz, D. Karunagaran, S. Lavi, B. J. Ratzkin, and Y. Yarden. 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.

Vivanco, I., and C. L. Sawyers. 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer **2**:489-501.

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.

Wang, L. M., A. Kuo, M. Alimandi, M. C. Veri, C. C. Lee, V. Kapoor, N. Ellmore, X. H. Chen, and J. H. Pierce. 1998. ErbB2 expression increases the spectrum and potency of ligand-mediated signal transduction through ErbB4. Proc Natl Acad Sci U S A **95**:6809-6814.

Wang, Y., S. Pennock, X. Chen, and Z. Wang. 2002. Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival. Mol Cell Biol **22**:7279-7290.

Wang, Z., L. Zhang, T. K. Yeung, and X. Chen. 1999. Endocytosis deficiency of epidermal growth factor (EGF) receptor-ErbB2 heterodimers in response to EGF stimulation. Mol Biol Cell **10**:1621-1636.

Waterman, H., M. Katz, C. Rubin, K. Shtiegman, S. Lavi, A. Elson, T. Jovin, and Y. Yarden. 2002. A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling. Embo J **21**:303-313.

Waterman, H., I. Sabanai, B. Geiger, and Y. Yarden. 1998. Alternative intracellular routing of ErbB receptors may determine signaling potency. J Biol Chem **273**:13819-13827.

Waugh, M. G., D. Lawson, and J. J. Hsuan. 1999. Epidermal growth factor receptor activation is localized within low-buoyant density, non-caveolar membrane domains. Biochem J **337 ( Pt 3)**:591-597.

Wendland, B. 2002. Epsins: adaptors in endocytosis? Nat Rev Mol Cell Biol **3**:971-977.

Williams, C. C., J. G. Allison, G. A. Vidal, M. E. Burow, B. S. Beckman, L. Marrero, and F. E. Jones. 2004. The ERBB4/HER4 receptor tyrosine kinase regulates gene expression by functioning as a STAT5A nuclear chaperone. J Cell Biol **167**:469-478.

Williams, E. E., L. J. Trout, R. M. Gallo, S. E. Pitfield, I. Bryant, D. J. Penington, and D. J. Riese, 2nd. 2003. A constitutively active ErbB4 mutant inhibits drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines. Cancer Lett **192**:67-74.

Wiseman, S. M., N. Makretsov, T. O. Nielsen, B. Gilks, E. Yorida, M. Cheang, D. Turbin, K. Gelmon, and D. G. Huntsman. 2005. Coexpression of the type 1 growth factor receptor family members HER-1, HER-2, and HER-3 has a synergistic negative prognostic effect on breast carcinoma survival. Cancer **103**:1770-1777.

Witton, C. J., J. R. Reeves, J. J. Going, T. G. Cooke, and J. M. Bartlett. 2003. Expression of the HER1-4 family of receptor tyrosine kinases in breast cancer. J Pathol **200**:290-297.

Wong, A. J., J. M. Ruppert, S. H. Bigner, C. H. Grzeschik, P. A. Humphrey, D. S. Bigner, and B. Vogelstein. 1992. Structural alterations of the epidermal growth factor receptor gene in human gliomas. Proc Natl Acad Sci U S A **89**:2965-2969.

Wong, E. S., C. W. Fong, J. Lim, P. Yusoff, B. C. Low, W. Y. Langdon, and G. R. Guy. 2002. Sprouty2 attenuates epidermal growth factor receptor ubiquitylation and endocytosis, and consequently enhances Ras/ERK signalling. Embo J **21**:4796-4808.

Worthylake, R., L. K. Opresko, and H. S. Wiley. 1999. ErbB-2 amplification inhibits down-regulation and induces constitutive activation of both ErbB-2 and epidermal growth factor receptors. J Biol Chem **274**:8865-8874.

Xia, L., L. Wang, A. S. Chung, S. S. Ivanov, M. Y. Ling, A. M. Dragoi, A. Platt, T. M. Gilmer, X. Y. Fu, and Y. E. Chin. 2002. Identification of both positive and negative domains within the epidermal growth factor receptor COOH-terminal region for signal transducer and activator of transcription (STAT) activation. J Biol Chem **277**:30716-30723.

Xu, W., M. Marcu, X. Yuan, E. Mimnaugh, C. Patterson, and L. Neckers. 2002. Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. Proc Natl Acad Sci U S A **99**:12847-12852.

Xu, W., E. Mimnaugh, M. F. Rosser, C. Nicchitta, M. Marcu, Y. Yarden, and L. Neckers. 2001. Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. J Biol Chem **276**:3702-3708.

Xu, W., X. Yuan, K. Beebe, Z. Xiang, and L. Neckers. 2007. Loss of Hsp90 association up-regulates Src-dependent ErbB2 activity. Mol Cell Biol **27**:220-228.

Yang, S., M. A. Raymond-Stintz, W. Ying, J. Zhang, D. S. Lidke, S. L. Steinberg, L. Williams, J. M. Oliver, and B. S. Wilson. 2007. Mapping ErbB receptors on breast cancer cell membranes during signal transduction. J Cell Sci.

Yarden, Y. 2001a. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. Eur J Cancer **37 Suppl 4**:S3-8.

Yarden, Y. 2001b. Biology of HER2 and its importance in breast cancer. Oncology **61 Suppl 2**:1-13.

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

Yen, L., Z. Cao, X. Wu, E. R. Ingalla, C. Baron, L. J. Young, J. P. Gregg, R. D. Cardiff, A. D. Borowsky, C. Sweeney, and K. L. Carraway, 3rd. 2006. Loss of Nrdp1 enhances ErbB2/ErbB3-dependent breast tumor cell growth. Cancer Res **66**:11279-11286.

Yu, X., K. D. Sharma, T. Takahashi, R. Iwamoto, and E. Mekada. 2002. Ligand-independent dimer formation of epidermal growth factor receptor (EGFR) is a step separable from ligand-induced EGFR signaling. Mol Biol Cell **13**:2547-2557.

Yuste, L., J. C. Montero, A. Esparis-Ogando, and A. Pandiella. 2005. Activation of ErbB2 by overexpression or by transmembrane neuregulin results in differential signaling and sensitivity to herceptin. Cancer Res **65**:6801-6810.

Zaczek, A., B. Brandt, and K. P. Bielawski. 2005. The diverse signaling network of EGFR, HER2, HER3 and HER4 tyrosine kinase receptors and the consequences for therapeutic approaches. Histol Histopathol **20**:1005-1015.

Zahnow, C. A. 2006. ErbB receptors and their ligands in the breast. Expert Rev Mol Med **8**:1-21.

Zandi, R., A. B. Larsen, P. Andersen, M. T. Stockhausen, and H. S. Poulsen. 2007. Mechanisms for oncogenic activation of the epidermal growth factor receptor. Cell Signal **19**:2013-2023.

Zhou, P., N. Fernandes, I. L. Dodge, A. L. Reddi, N. Rao, H. Safran, T. A. DiPetrillo, D. E. Wazer, V. Band, and H. Band. 2003. ErbB2 degradation mediated by the co-chaperone protein CHIP. J Biol Chem **278**:13829-13837.

Zhou, W., and G. Carpenter. 2001. Heregulin-dependent translocation and hyperphosphorylation of ErbB-2. Oncogene **20**:3918-3920.

Zimonjic, D. B., M. Alimandi, T. Miki, N. C. Popescu, and M. H. Kraus. 1995. Localization of the human HER4/erbB-4 gene to chromosome 2. Oncogene **10**:1235-1237.

Zoncu, R., R. M. Perera, R. Sebastian, F. Nakatsu, H. Chen, T. Balla, G. Ayala, D. Toomre, and P. V. De Camilli. 2007. Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. Proc Natl Acad Sci U S A **104**:3793-3798.