Molecular Mechanisms Controlling Endocytic Downregulation of EGFR and ErbB3

By

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2012

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Financial support provided by
The South-Eastern Norway Regional Health Authority and Inven2

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Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1497

ISBN 978-82-8264-218-7

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ACKNOWLEDGMENTS

The work presented in this dissertation has been carried out at the Laboratory for Molecular and Cellular Cancer Research at the Institute of Clinical Medicine, University of Oslo/ Department of Pathology, Division of Diagnostics and Intervention, Oslo University Hospital, during the period 2007-2012. This work was funded by the South-Eastern Norway Regional Health Authority and Inven2, to whom I am truly grateful.

I would like to express my gratitude to my supervisor Prof. Dr. Inger Helene Madshus and my co-supervisor Dr. Espen Stang for their patient guidance and support. I am grateful to Inger Helene for giving me the opportunity to start the PhD program and to complete my studies by providing additional funding. I appreciate her kindness, expertise and valuable feedback over time. I am deeply thankful to Espen, for his thoughtful work, expertise, constructive ideas and insightful comments on the manuscripts and this thesis. His attention to detail, honest objective feedback and always being available for discussions are irreplaceable.

My gratitude goes out to everyone else who devoted time and knowledge to the work presented here. Your contribution was priceless. Thanks to all my co-authors whose hard work made writing this thesis possible. I am grateful to Kamilla Breen and Sissel B. Rønning, with whom I worked on the early project, for their precious experiments and kindness. Many tanks to Monika Szymanska for her recent work and willingness to help that were invaluable. Vibeke Bertelsen, with whom I collaborated on several projects, has greatly contributed with her precise experiments, critical thinking and numerous discussions. I thank her for providing valuable comments on the manuscripts and this thesis, for patiently answering “thousands of questions” and helping me understand the value of our research. Vibeke has also been a great travel and conference companion, and a wonderful friend. Special thanks to Marianne S. Rødland for her constant assistance in both scientific and non-scientific way, as well as her friendship and warmth throughout the years. My thanks also go to the other past and present members of my research group, especially Juliana B. Hughes, Michael V. Grandal, Tram T. Vuong, Christian Berger and Anne Marthe Fosdahl. It was a true pleasure to be at work with all of you around.

I am very grateful to all the people at the institute, including the administrative staff, for creating a truly friendly and relaxed working environment. I appreciate the professional and mental support that you generously provided numerous times. Thanks to all my office mates and coffee/lunch companions within and outside the institute, for the inspiring talks, laughter and many precious moments that I will keep with me.

I would like to thank, from the bottom of my heart, my dearest wonderful family and friends for their endless support and belief in me. All of you were tireless cheerleaders of this work, encouraging me to stay motivated and fight for what I wanted to accomplish. Thank you!

Oslo, September 2012

Malgorzata Magdalena Sak

TABLE OF CONTENTS

ABBREVIATIONS .... **II**
PAPERS INCLUDED .... **III**
INTRODUCTION **1**
The ErbB protein family and signal transduction 1
Structural properties 2
ErbB activation and signaling 4
Endocytic transport 9
Protein localization and trafficking 9
Closer look at endocytosis 11
Endocytic transport of ErbB proteins 16
ErbB proteins in cancer and anti-ErbB therapy 22
ErbB proteins in cancer 22
Anti-ErbB therapeutics 24
Resistance to anti-ErbB therapy 26
AIMS OF THE STUDY **29**
SUMMARY OF PAPERS **31**
Paper I 31
Paper II 32
Paper III 33
METHODOLOGICAL CONSIDERATIONS **35**
Experimental model 35
Choice of cell lines and stable transfection 35
Stimulation with ligands 36
Transient protein expression 36
Protein knock-down by RNA interference 37
Biochemical methods 38
Antibody specificity 38
Kinase inhibitor specificity 38
Western blot, Immunoprecipitation (IP), Co-IP, Biotinylation 39
Immunofluorescence, flow cytometry and confocal microscopy 40
Immuno- electron microscopy (Immuno-EM) 41
Internalization of radio-labeled ligand 42
GENERAL DISCUSSION **43**
Ubiquitination of EGFR as a signal for endocytosis 43
Endocytosis of ErbB3 45
Reduced endocytosis of EGFR upon dimerization with ErbB3 46
Inhibitory effect of ErbB2 on endocytic downregulation of ErbB3 47
Inhibition of HRG-signaling and induction of ErbB3 downregulation by pertuzumab in cells co-expressing ErbB2 48
ErbB3 kinase activity and signaling 49
Concluding remarks 50
REFERENCES **52**
PAPERS I-III

ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
ADCC	Antibody-dependent cellular cytotoxisity
AP2	Adaptor protein complex 2
Cbl	Casitas B-lineage lymphoma
CCP	Clathrin coated pit
CCV	Clathrin coated vesicle
CIE	Clathrin independent endocytosis
CLASP	Clathrin-associated sorting protein
CME	Clathrin mediated endocytosis
DUB	Deubiquitinating enzyme
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Eps15	Epidermal growth factor receptor substrate 15
ESCRT	Endosomal sorting complex required for transport
GA	Geldanamycin
GPCR	G-protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
HRG	Heregulin
HRG-ECD	Heregulin-extracellular domain
Hrs	Hepatocyte-growth-factor-regulated tyrosine-kinase substrate
Hsp90	Heat shock protein 90
ILV	Intraluminal vesicle
kDa	Kilodalton
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
Lys	Lysine
mAb	Monoclonal antibody
MAPK	Ras/mitogen-activated protein kinase
mRNA	Messenger RNA
MVB	Multivesicular body
Nrdp1	Neuregulin receptor degradation pathway protein 1
PAE	Porcine aortic endothelial
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinositol 3 kinase
RTK	Receptor tyrosine kinase
siRNA	Small interfering RNA
STAM	Signal transducing adaptor molecule
Tf	Transferrin
TfR	Transferrin receptor
TGF-α	Transforming growth factor-α
TKI	Tyrosine kinase inhibitor
Ub	Ubiquitin
UIM	Ubiquitin-interacting motif

PAPERS INCLUDED

Paper I Vibeke Bertelsen, Malgorzata Magdalena Sak, Kamilla Breen, Marianne S. Rødland, Lene E. Johannessen, Linton M. Traub, Espen Stang, and Inger Helene Madshus

A chimeric pre-ubiquitinated EGF Receptor is constitutively endocytosed in a clathrin-dependent, but kinase-independent manner.

Traffic, Vol. 12(4), 507-520, April 2011.

Paper II Malgorzata Magdalena Sak*, Kamilla Breen*, Sissel Beate Rønning, Nina Marie Pedersen, Vibeke Bertelsen, Espen Stang, and Inger Helene Madshus

*These authors contributed equally to this work

The oncoprotein ErbB3 is endocytosed in the absence of added ligand in a clathrin-dependent manner.

Carcinogenesis, Vol. 33(5), 1031-1039, May 2012.

Paper III Malgorzata Magdalena Sak, Monika Szymanska, Vibeke Bertelsen, Max Hasmann, Espen Stang, and Inger Helene Madshus

Pertuzumab counteracts the inhibitory effect of ErbB2 on degradation of ErbB3.

Submitted (Carcinogenesis)

INTRODUCTION

Cells are constantly dependent on external signals for survival, growth, differentiation and migration. A multicellular organism can function because cells communicate with each other throughout the body. Part of this communication is conveyed by ligands, which are small chemicals, peptides, or proteins that often travel long distances to bind with certain affinity to their respective receptors. This binding initiates a response in form of signaling cascades that induce alterations in gene transcription and result in cellular and tissue changes. ErbB proteins are a subfamily of receptor tyrosine kinases (RTKs) localized to the cell surface, where upon ligand (growth factor) binding, they trigger intracellular responses. Their physiological function is regulating embryonic development but also tissue renewal and repair throughout the lifespan of an organism. Tissue homeostasis is maintained thanks to, among other things, the tight regulation and integration of ErbB signaling (Figure 1), and uncontrolled survival, growth and mobility of cells often leads to tumorigenesis. Downregulation of receptors by internalization and subsequent degradations is one of negative feedback loops developed by cells to regulate ErbB signaling. Understanding mechanisms of receptor downregulation and other regulatory processes provides the foundation for advancement in cancer screening, diagnosis, treatment and prevention. This thesis aims at exploring some aspects of how ErbB proteins influence each other, as well as how their signaling and endocytic downregulation is modulated both physiologically and therapeutically.

The ErbB protein family and signal transduction

Human RTKs contain 20 subfamilies of single-spanning transmembrane proteins that share similar overall structure and activation mechanism (reviewed in Lemmon and Schlessinger 2010). ErbB proteins were named so because of homology to the erythroblastic leukemia viral oncogene (*v-erbB*, avian) and the receptor family have four closely related members: epidermal growth factor (EGF) receptor (EGFR, also called ErbB1 or HER1), ErbB2 (Neu/HER2), ErbB3 (HER3) and ErbB4 (HER4). ErbB proteins are expressed in a number of different tissues of epithelial, mesenchymal and neuronal origin and they regulate survival, growth and differentiation during embryogenesis and in the adult organism, including maintaining skin, development and maintaining cardiovascular and nervous systems and mammary gland (reviewed in Olayioye et al. 2000; and in Eccles 2011). A number of ligands have been found to bind to the ErbB proteins (see Figure 2). Those ligands can be synthesized by distantly localized cells and travel throughout body fluids, or they can be locally available, and their spatial and

temporal expression is strictly regulated (Figure 1 and reviewed in Olayioye et al. 2000). Their production is also integrated with other cellular pathways, like for instance, G-protein-coupled receptor (GPCR) or estrogen receptor signaling that induce ADAM (A disintegrin and metalloproteinase)-mediated cleavage of precursor ErbB ligand (Figure 1).

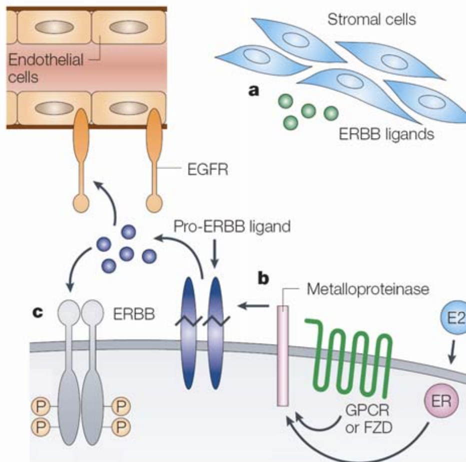


Figure 1. Activation of ErbB proteins by autocrine and paracrine ligands. a) Paracrine ErbB ligands (green circles) are released from stromal cells. **b)** Autocrine ligand (blue circles) production results from the activation of GPCRs, Fizzled (FZD), or estradiol (E2)-bound estrogen receptor (ER), causing the metalloproteinase-mediated cleavage and release of pro-EGF-related ligands by ectodomain shedding. **c)** Ligand binding causes ErbB kinase activation and phosphorylation (P). Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Hynes and Lane 2005), © 2005. The figure legend is modified.

Structural properties

Overall structure of ErbB members is shown in Figure 2. Each receptor is composed of three functional domains: the amino (N)-terminal extracellular domain, the α-helical transmembrane segment, and the carboxy (C)-terminal intracellular tyrosine kinase domain. The N-terminal domain is highly glycosylated and consists of subdomains I and III that bind ligand, and cysteine-rich subdomains II and IV that are involved in dimerization (reviewed in Burgess et al. 2003). Subdomain II contains a "dimerization arm" that constitutes the major part of the dimerization interface. In the absence of ligand, the extracellular domain of EGFR, ErbB3 and ErbB4 exists in a tethered 'closed' conformation, in which the dimerization arm is not available for interaction with dimerization partners (Figure 2), this being one of auto-inhibitory mechanisms (Ferguson et al. 2003). The single-span transmembrane region connects the extracellular domain with the intracellular domain that contains the tyrosine kinase and the C-terminal tail. The C-terminal tail has an additional auto-inhibitory function in the absence of stimuli (see section about allosteric activation of ErbB proteins below). Intra- and extracellular juxtamembrane domains are the regions situated in close proximity to the plasma membrane.

Despite the structural similarities, the ErbB proteins vary with respect to their ligand specificity, kinase activity and signaling activation pattern. EGFR is a 170 kilodalton (kDa) protein and was the first ErbB protein to be discovered as a receptor for the previously characterized EGF (Carpenter et al. 1978).

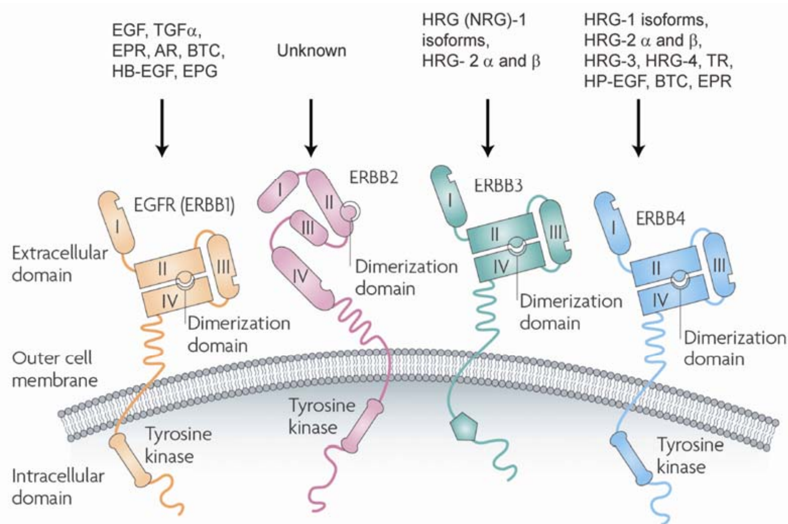


Figure 2. ErbB receptors and their ligands. Each member of ErbB family, except ErbB2, binds a distinct subset of ligands: EGF, transforming growth factor-α (TGF-α), epiregulin (EPR), amphiregulin (AP), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), epigen (EPG), heregulin (HRG) (also known as neuregulin (NRG)), and tomoregulin (TR). Each receptor is composed of the N-terminal extracellular domain, the transmembrane segment, and the C-terminal intracellular tyrosine kinase domain. The extracellular subdomains I and III that bind ligand, and subdomains II and IV that are involved in dimerization (reviewed in Burgess et al. 2003). In the absence of ligand, EGFR, ErbB3 and ErbB4 exist in a 'closed' conformation in which the dimerization arm is 'buried' within the intramolecular tether. ErbB2 exists in an 'open' conformation, continuously available for dimerization. ErbB3 has a marginal tyrosine kinase activity, compared to other ErbB family members. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Baselga and Swain 2009), © 2009. The figure legend is modified.

The major parts of its structure has been determined by crystallography and negative-stain electron microscopy (Bessman and Lemmon 2012). Glycosylation of its extracellular domain has been shown to affect ligand binding, association with other ErbB proteins and with gangliosides (Tsuda et al. 2000; Whitson et al. 2005; Yoon et al. 2006; Kawashima et al. 2009). ErbB2 has a molecular weight of 185 kDa. No ligand has yet been found for this orphan receptor and while all other ErbB proteins have a tethered conformation, the extracellular domain of ErbB2 appears to have an extended 'open' conformation, with its dimerization arm continuously exposed (Figure 2 and Cho et al. 2003; Garrett et al. 2003; Vicente-Alique et al. 2011). However, lack of ErbB2 auto-inhibition has been questioned based on the studies of *Drosophila melanogaster* EGFR (dEGFR) that is structurally similar to ErbB2. Even though dEGFR, like ErbB2, lacks the intramolecular tether, it is held inactive by a set of distinct auto-inhibitory interactions and becomes activated by ligand, which suggests existence of similar activation mechanism for ErbB2 (Alvarado et al. 2009). ErbB2's unique structure makes it a favorable

dimerization partner and when overexpressed, ErbB2 can constitutively self-associate or associate with other ErbB proteins and become activated in a ligand-independent manner (Yuste et al. 2005; Yang et al. 2007; Junttila et al. 2009). ErbB3 is a 180 kDa protein. Its extracellular domain structure has been described by crystallography and electron microscopy studies (Cho and Leahy 2002; Vicente-Alique et al. 2011). ErbB3 was long considered kinase-dead because it lacks certain amino acid residues important for kinase activity (Guy et al. 1994; Sierke et al. 1997; Jura et al. 2009). It has, however, recently been postulated that ErbB3 has a weak kinase activity, but depends on heterodimerization for efficient activation (Yang et al. 2007; Shi et al. 2010; Telesco et al. 2011) and based on chimeric studies, the C-terminal tail of ErbB3 has been proposed to lack the auto-inhibitory ability (Bublil et al. 2010). ErbB4, a 180 kDa protein, is encoded by one gene (Zimonjic et al. 1995), but alternative messenger RNA (mRNA) splicing gives rise to several functionally distinct isoforms of ErbB4 (reviewed in Junttila et al. 2000). The crystal structure of its extracellular region has been described (Bouyain et al. 2005), however it is the least well characterized member of the ErbB protein family.

ErbB activation and signaling

Ligand binding to the ErbB proteins induces their dimerization and activation of their cytoplasmic kinases followed by initiation of signaling pathways. Variety and specificity of ErbB signaling partly comes from the distinct ligands available, formation of distinct homo/heterodimers, as well as from cellular and subcellular context, meaning availability of signaling molecules that are expressed and localized to the signaling site within the cell.

Allosteric activation of ErbB proteins

Ligand binding to domain I (and III) induces a conformational change in the tethered receptor, exposing the dimerization arm (II), stabilizing the extended conformation of the receptor and allowing for receptor dimerization (Figure 3 and reviewed in Burgess et al. 2003; Dawson et al. 2007). As a consequence of conformational changes in the extracellular and transmembrane domains, the intracellular kinase domain undergoes allosteric activation through formation of an asymmetric dimer, with the N-terminal lobe of one tyrosine kinase interacting with the C-terminal lobe of the other kinase (Figure 3 and Zhang et al. 2006). Helix-helix interactions in the transmembrane domain participate in dimer stabilization, since the alpha helix tends to self-associate (reviewed in Cymer and Schneider 2010). Also, intracellular juxtamembrane region and the C-terminal tail have been reported to regulate the allosteric kinase activation (reviewed in Bose and Zhang 2009). The C-terminal tail has an auto-inhibitory function in an inactive ErbB (except ErbB3, see the previous section) and deletions in the C-

terminal tail have been demonstrated to render EGFR constitutively active (reviewed in Pines et al. 2010). Upon activation, the tyrosine kinase mediates autophosphorylation of conserved tyrosine residues in the C-terminal tail of the other receptor, but details of this activation model have been discussed. It has been postulated that at equilibrium, 3-20 % unliganded EGFR has an "open" conformation (Ferguson et al. 2003). EGFR dynamically fluctuates between unoccupied monomers and unoccupied dimers and ligand binding shifts monomer-dimer equilibrium favoring dimerization. A negative cooperativity model has been proposed for binding of ligand to EGFR, where binding of a second ligand to the dimer is weaker, then binding of the first ligand (reviewed in Lemmon 2009). Another view is that EGFR exists in preformed inactive oligomers and of which activation could depend on more subtle conformational changes (reviewed in Bessman and Lemmon 2012). Recent studies reported formation of inactive dimers in absence of ligand and independent of kinase activity, and ligand binding has been proposed to stabilize the dimers and promote kinase activation (Low-Nam et al. 2011; Macdonald-Obermann et al. 2011). Some of the contradictory results from studies of the mechanism of ErbB activation could come from the differences in methods and experimental system used.

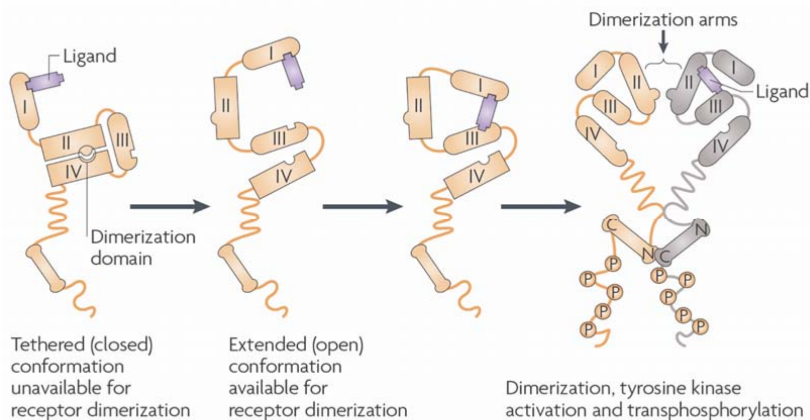


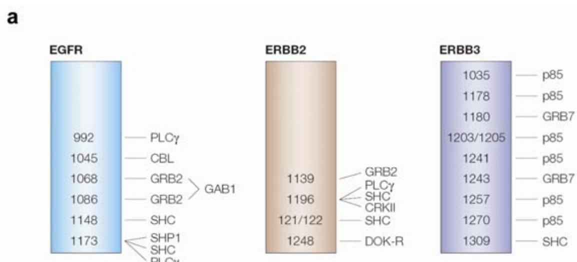
Figure 3. Activation of ErbB receptors. Ligand binding mediates rearrangements in the extracellular domain followed by exposure of the dimerization arm (in subdomain II), receptor dimerization, tyrosine kinase activation and phosphorylation of the tyrosine residues in receptors' C-terminal tails. The kinase domain interaction is asymmetric, with the N-terminal lobe of one tyrosine kinase interacting with the C-terminal lobe of the other kinase. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Baselga and Swain 2009), © 2009. The figure legend is modified.

Main ErbB regulated signaling pathways and cross-talk with other pathways

The signaling pathways are based on the interplay of signaling proteins, adaptor and scaffold proteins, and second messengers. Protein-protein and protein-lipid interactions are modulated by post-translational modifications, such as protein or lipid phosphorylation, protein ubiquitination, and cleavage events and are all mediated by specific enzymes. These, often transient, modifications regulate signaling through localizing proteins to the plasma membrane and/or protein complexes at the site where the signaling takes place. Such interactions are mediated because a number of different conserved domains present in a protein can specifically recognize various protein sequence motifs, phospholipids, phosphotyrosines, ubiquitin (Ub) moieties or other modifications (reviewed in Pawson and Nash 2003). Upon ErbB activation, conserved tyrosines in the C-terminal tails of both receptors in a dimer become phosphorylated and these phosphotyrosines serve as docking sites for downstream effectors containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Figure 4 a).

ErbB signaling network is complicated and involves several signaling pathways. EGFR-mediated signaling is illustrated as an example in Figure 4 b. Among main ErbB activated pathways we find: the Phospholipase C γ (PLC γ) pathway, the Ras/mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol (PI) 3 kinase (PI3K) pathway and the Signal transducer and activator of transcription (STAT) pathway, leading to different cellular outcomes, like cell survival, proliferation, differentiation, migration or apoptosis (Figure 4 b and reviewed in Yarden and Sliwkowski 2001). Not all pathways are activated simultaneously. Specific activation of different signaling pathways and the fate of receptors depend on the ligand (and ligand affinity), composition of heterodimers and on the cellular context. It has for instance been demonstrated that different ligands result in differential activation of EGFR (McCole et al. 2007), and low affinity ligands have in turn been reported to rescue receptors from degradative pathways (French et al. 1995; Longva et al. 2002). Different phosphotyrosine patterns of each receptor resulting in recruitment of different effectors (Figure 4 a) accounts for variety of signaling pathways that come from homo/heterodimers. ErbB3's oncogenic potency partly resides in its intracellular domain which, in contrast to other ErbB proteins, contains multiple sites for direct binding of the p85 subunit of PI3K (Hellyer et al. 1998), however there are several mechanisms that seem to restrain ErbB3 signaling. For instance, N-glycosylation of the extracellular domain prevents spontaneous dimerization and tumor formation (Yokoe et al. 2007; Takahashi et al. 2008). The ability of ErbB receptors to form homo- and heterodimers has, however, been disputed. Ligand-induced homodimers have been reported for the EGFR, but ErbB2 and ErbB3 were initially thought incompetent with respect to formation of active homodimers (reviewed in Burgess et al. 2003; Berger et al. 2004). ErbB2 homodimers have later been detected upon overexpression of

ErbB2 (Yang et al. 2007). Both dimerization and oligomerization of the full-length ErbB3 and its soluble extracellular domain has been demonstrated (Landgraf and Eisenberg 2000; Kani et al. 2005). However, the ErbB3 homodimers and oligomers have been considered inactive due to the impaired kinase activity of ErbB3 and ErbB3 oligomers dissociate upon heregulin (HRG) binding (Kani et al. 2005). ErbB2 overexpression correlates with increased proliferation, differentiation and migration (reviewed in Olayioye et al. 2000). ErbB2 has in addition been demonstrated to increase the affinity that its dimerization partners have for their ligands (Citri et al. 2003). ErbB2 is thought to be the preferred dimerization partner, (Graus-Porta et al. 1997) and ErbB2-ErbB3 heterodimers, which upon activation have a strong anti-apoptotic and proliferative capacity, has been considered the most potent signaling unit (reviewed in Sliwkowski et al. 1994; Citri et al. 2003). It has moreover been demonstrated that upon ErbB2 overexpression, ErbB2-ErbB3 can heterodimerize in a ligand-independent manner (Junttila et al. 2009).



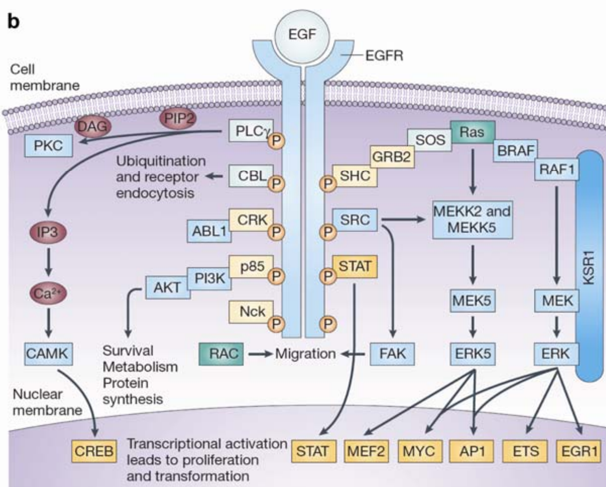


Figure 4. Phosphorylation sites of ErbB C-terminal tail and ErbB signaling network. a) Schematic representation of the main autophosphorylation sites (tyrosine residues) in EGFR, ErbB2 and ErbB3 that serve as docking sites for a range of the signaling molecules, indicated to the right of those sites. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Hynes and Lane 2005), © 2005. **b)** Signaling proteins dock onto EGFR's phosphorylated sites and initiate various signaling cascades that transduce EGF signals to generate specific biological responses. The ErbB signaling network has been reviewed in detail elsewhere (Yarden and Sliwkowski 2001; Citri and Yarden 2006). Best characterized downstream pathways, including the MAPK, PI3K, PLCγ and STAT pathways, are shown here. Kinases are in blue, scaffolds are in dark blue, adaptor proteins are in yellow, G proteins are in green, small molecule second messengers are in purple. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Kolch and Pitt 2010), © 2010.The figure legends are modified.

Signaling diversification is additionally modulated by other pathways that trans-regulate or are involved in cross-talk with ErbB signaling pathways, both under normal cell growth and in transformed cells (reviewed in Avraham and Yarden 2011). For instance, the kinase Src was demonstrated to phosphorylate ErbB proteins and regulate their function (Biscardi et al. 1999; Contessa et al. 2006; Xu et al. 2007). Cross-talk between HRG signaling and GPCR 30 has been indicated in proliferation and migration of cancer cells and in resistance to antitumor therapy (Ruan et al. 2012a; Ruan et al. 2012b). ErbB protein cross-talk with c-Met receptor has been further reported to drive resistance to anti-cancer agents (reviewed in Arteaga 2007; Karamouzis et al. 2009). ErbB signaling has also been demonstrated to cross-react with nuclear factor kappaB (NF-κB) and insulin-like growth factor type I receptor (IGF-1R) pathways (Chen et al. 2003; Knowlden et al. 2011). Likewise has integrin and E-cadherin-mediated EGFR activation been reported (Bill et al. 2004; Shen and Kramer 2004).

Microenvironment

ErbB proteins reside in the plasma membrane of which molecular composition plays a crucial role in modulating ErbB activation. Some of the evidence comes from the studies demonstrating that EGFR ligand binding and activation was different in detergent micelles than it was in the membrane context (reviewed in Bessman and Lemmon 2012). Plasma membrane is a noncovalent assembly of lipids (saturated, non-saturated fatty acids, cholesterol, sphingolipids, and PIs) into a lipid bilayer with various proteins held by noncovalent interactions. PIs, because of their different phosphorylation patterns and specific distribution among cellular compartments, are thought to serve as landmarks for binding of signaling, scaffold, adaptor and regulatory proteins (reviewed in Wenk and De Camilli 2004). In addition to glycosylation of ErbB proteins, glycosylation of both lipids (glycosphingolipids) and other proteins (glycoproteins) present in the plasma membrane can as well affect ErbB signaling. For instance, the glycoprotein mucin, Muc4, has been shown to regulate ErbB2-ErbB3 mediated signaling (Carraway et al. 2009) and gangliosides have been observed to affect kinase activity of EGFR (Coskun et al. 2011). It is now well established that transmembrane receptors are neither uniformly distributed nor uniformly activated (Casaletto and McClatchey 2012). Spatial organization of receptors within the cell, receptors' *cis*-interactions in the cell membrane and their intercellular *trans*-interactions, as well as interaction with membrane lipids and other molecules, all contribute to formation of different signaling foci and activation of different signaling pathways (reviewed in Bethani et al. 2010; and in Casaletto and McClatchey 2012). The plasma membrane is not a homogenous structure but contains "floating" domains that are more structurally organized. Lipid membrane rafts have been defined as "small (10-200 nm) heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes" (Pike 2006). A line of evidence suggests that ErbB proteins are localized to such

lipid rafts and that lipid rafts regulate ErbB signal transduction, by enrichment of signaling molecules (reviewed in Lambert et al. 2009). The EGFR has been reported to localize to caveolae, a specialized type of lipid rafts, in their unstimulated state and to migrate out of caveolae upon EGF stimulation (Mineo et al. 1999). However, others have shown that EGFR was not concentrated in caveolae and its localization to caveolae was not changed upon incubation with EGF (Ringerike et al. 2002).

Attenuation of signaling

Both qualitative regulation and temporal regulation of signaling is an important factor in determining biological output. Several mechanisms work to orchestrate attenuation of ErbB signaling and those are classified as immediate or late feedback loops. The immediate attenuation mechanisms include: modifications of pathway components (attachment or removal of phosphate groups or small regulatory proteins, like Ub), secondary (or backwards) phosphorylation of upstream components within the signaling pathway, microRNA-mediated regulation of protein expression, as well as receptor endocytosis and subsequent degradation (reviewed in Avraham and Yarden 2011). Late feedback loops comprise regulation of *de novo* synthesis of various negative regulators, like inhibitors, transcriptional repressors or phosphatases. For example, EGFR and ErbB2 activation has been demonstrated to induce expression of mitogen-induced gene 6 (MIG6, also called RALT) that binds to the intracellular part of the receptors, inhibits their activity and promotes their endocytosis and degradation (reviewed in Bose and Zhang 2009; Frosi et al. 2010). Endocytic downregulation and degradation are discussed in more details in the following section.

Endocytic transport

Protein localization and trafficking

Localization of proteins to discrete subcellular regions has a crucial regulatory function. Such compartmentalization includes localization to different organelles (e. g. plasma membrane, intracellular membranous structures or nucleus), and further into different microdomains, like the earlier discussed lipid rafts. Cell polarity is one way in which a cell spatially can regulate RTK signaling, and loss of cell polarization is one hallmark of cancer (reviewed in Casaletto and McClatchey 2012). EGFR and ErbB2 have been found localized mainly to the basolateral membrane in polarized cells (reviewed in Sorkin and Goh 2008). Membrane localized proteins traffic throughout the cells by membrane trafficking, a vesicular transport that includes, among others, exocytosis, endocytosis, transcytosis, recycling and sorting to degradative pathways. The endocytic system is a network of those complex and

interconnected trafficking pathways between the plasma membrane and endomembrane organelles. It is, just like signaling, strictly regulated by protein-protein and protein-lipid interactions. PIs can be phosphorylated on different sites within the inositol ring giving rise to seven different PI species. Each PI interacts with proteins that contain respective PI-specific lipid-binding domains, and because the distribution of the different PIs is restricted to specific subcellular regions, PIs control localization of PI-binding proteins to different organelles (reviewed in Wenk and De Camilli 2004; and in Le Roy and Wrana 2005). Phosphorylation of PIs is regulated by PI kinases and phosphatases and their short-lived character allows for rapid shift of membrane identity (reviewed in Krauss and Haucke 2009). As an example, sorting nexins (SNXs) are proteins that bind PIs and their function is to regulate endocytosis, endosomal sorting and endosomal signaling (reviewed in Cullen 2008). Membrane identity is also controlled by small GTPases called Rab proteins. Rab proteins are reversibly associated with specific cellular compartments and through recruitment of various effector proteins they regulate various steps of vesicular trafficking (reviewed in Stenmark 2009).

Cargo, destined for endocytosis, are internalized into vesicles, called early endosomes, from where they are either recycled back to the plasma membrane, sorted to late endosomes and committed to the lysosomal degradation pathway, or delivered to the *trans*-Golgi network (reviewed in Jovic et al. 2010). Endocytosis can function in bringing nutrition into cells and also regulates size and composition of the plasma membrane. Endocytosis and recycling process that brings nutrition into the cell is illustrated by both the transferrin (Tf) receptor (TfR) and the low-density lipoprotein (LDL) receptor (LDLR). When bound cargo, iron (Fe^{3+}) or LDL respectively, they get endocytosed, release their cargo inside the endosome, and then TfR with bound Tf and LDLR are recycled back to the plasma membrane, ready for another round of endocytosis (reviewed in Maxfield and McGraw 2004). Endocytosed proteins can alternatively be degraded and such endocytic degradation is one of mechanisms the cells use to regulate abundance of receptors at the cell surface and to attenuate signaling. In such case, proteins localized to the limiting membrane of early endosomes are further internalized into intraluminal vesicles (ILVs) to form multivesicular bodies (MVBs) which further on mature into or fuse with lysosomes where degradation takes part. Sorting into MVBs generally occurs through interaction with the endosomal sorting complex required for transport (ESCRT) (reviewed in Raiborg and Stenmark 2009). In addition to lysosomal degradation, an alternative pathway of receptor downregulation has been reported, in which a receptor undergoes intramembrane proteolytic cleavage, followed by proteasomal degradation of generated fragments (Foveau et al. 2009). In case of signaling receptors, it is now widely established that endocytosis does not merely act in signal attenuation. It has been demonstrated that endocytic compartments also function as signaling platforms (reviewed in Hupalowska and Miaczynska 2012). There have been reports about the qualitative differences between EGFR signaling complexes

assembled at the cell surface and on endosomes (Burke et al. 2001; Wang et al. 2002). While cell surface EGFR was observed to mediate proliferative signaling, EGFR on the limiting membrane of endosomes was found to activate apoptosis (Hyatt and Ceresa 2008; Rush et al. 2012). Besides endocytic compartments, EGFR can localize to other organelles (reviewed in Han and Lo 2012). Full length, or cleaved ErbB proteins were found to shuffle to the nucleus, where they act as transcriptional regulators and nuclear localization is often linked to oncogenesis and therapeutic resistance (reviewed in Han and Lo 2012; and in Wang and Hung 2012). For instance, translocation of the full length EGFR from the plasma membrane to the nucleus has been proposed to involve translocation of EGFR through protein pore complexes (Liao and Carpenter 2007; Wang et al. 2012b).

Closer look at endocytosis

Endocytosis brings nutrition and other important molecules into the cell, regulates the composition of the plasma membrane and communication of the cell with the external environment. Accumulating evidence has indicated also other functions of endocytosis including mitosis, cell migration, immune response (antigen presentation and uptake of pathogens) and, as discussed above, cell signaling, (reviewed in Doherty and McMahon 2009). Endocytic mechanisms have also been hijacked by pathogens/toxins upon infection (Olsnes et al. 1985; Sandvig and van Deurs 2008).

Types of endocytosis

Endocytosis can generally be divided in phagocytosis and pinocytosis and the latter includes macropinocytosis and other types of pinocytosis, classified by their dependence or independence of clathrin, caveolin, and/or dynamin (Figure 5). While pinocytosis, the "fluid endocytosis", takes place in basically all cell types, phagocytosis is limited to phagocytes and is the way cells engulf big solid particles, like bacteria, into phagosomes. Macropinocytosis is a form of pinocytosis, through which cells take up bulk fluid and solid cargo. It is an actin-dependent, usually growth factor-regulated process, upon which surface membrane ruffles "close back" on the cell membrane giving rise to macropinosomes (reviewed in Lim and Gleeson 2010). The remaining types of pinocytosis are more selective when it comes to the cargo they take up and involve inward budding of the plasma membrane to form intracellular vesicles. Clathrin mediated endocytosis (CME), which depends on dynamin, is the best characterized type of pinocytosis. Clathrin independent endocytosis (CIE) is less well characterized, but includes several different pathways such as RhoA- (dynamin-dependent) or Cdc42- (dynamin-independent) mediated endocytosis, Arf6- or flotillin-dependent endocytosis (for which both dynamin-

dependence and dynamin-independence have been reported), and caveolin-dependent (dynamin-dependent) endocytosis (reviewed in Sandvig et al. 2011).

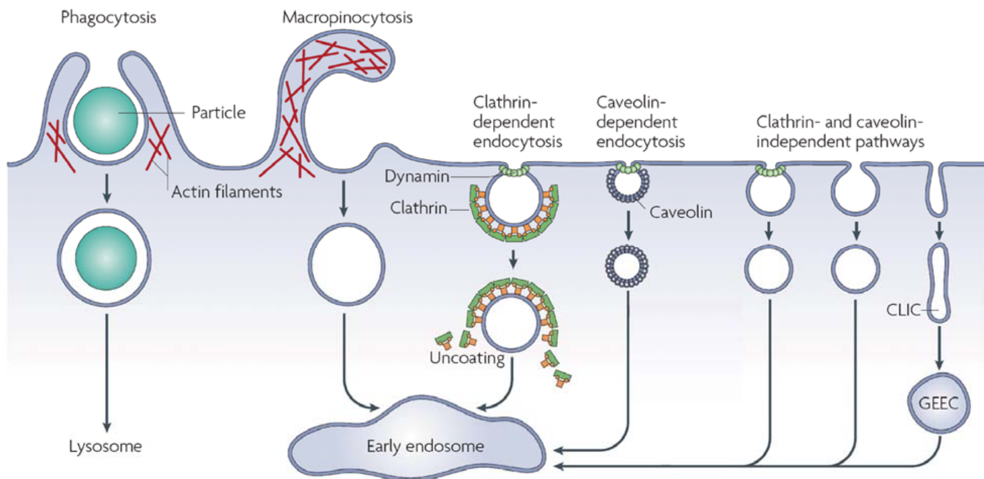


Figure 5. Pathways of entry into cells. Large particles can be taken up by phagocytosis and transported to lysosomes, whereas fluid uptake occurs by macropinocytosis. Both processes are dependent on a large scale actin-mediated remodeling of the plasma membrane. The size of such vesicles is much larger, compared to other endocytic pathways. Smaller particles are taken up by invagination of the plasma membrane and the formation of vesicles. Those pathways are clathrin- and dynamin-dependent, caveolin- and/or dynamin-dependent, or clathrin-, caveolin- and dynamin-independent. Most internalized cargos are delivered to early endosomes via vesicular (clathrin- or caveolin-coated vesicles), or tubular intermediates (clathrin-and dynamin-independent carriers (CLICs)). Some pathways may first traffic to glycosyl phosphatidylinositol-anchored protein enriched early endosomal compartments (GEEC), before they enter early endosomes. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Mayor and Pagano 2007), © 2007. The figure legend is modified.

Clathrin mediated endocytosis

CME is used by all eukaryotic cells and is generally a well conserved endocytic pathway starting from plants, through yeast to humans. In higher organisms, in addition to receptor/nutrient uptake and signal regulation, it functions in synaptic vesicle recycling in neurons and is used by pathogens as a cell entry mechanism (reviewed in McMahon and Boucrot 2011). CME can be constitutive, like for LDLR and TfR that are internalized and recycled, or induced by ligand (receptor mediated), like endocytosis of many GPCRs and RTKs that often results in lysosomal degradation. During CME, transmembrane receptors are recognized by intracellular adaptor proteins and packaged into clathrin coated pits (CCPs)

and subsequently internalized into clathrin coated vesicles (CCV). This process is called a CCV cycle and comprises several steps: nucleation, cargo selection, coat assembly, scission and uncoating (reviewed in McMahon and Boucrot 2011). Clathrin, the critical component of the clathrin coat, is a protein triskelion build up of three heavy chains and three light chains (reviewed in Kirchhausen 2000). The clathrin heavy chain contains binding sites for several other proteins, including endocytic adaptors, and clathrin light chain binds proteins that recruit the actin machinery. Another major component of CCP, adaptor protein complex 2 (AP2), is a heterotetrameric protein adaptor made of 4 non-identical subunits: α-subunit that binds cargo, other clathrin adaptors, accessory proteins and the plasma membrane through PI 4,5-bisphosphate (PIP_2), β2-subunit that binds clathrin heavy chain, other clathrin adaptors and accessory proteins, μ2-subunit that binds cargo and PIP_2, and the σ2-subunit that binds cargo (Collins et al. 2002; reviewed in Traub 2009). AP2 is activated by an AP2 associated kinase 1 (AAK1) and helps nucleation by simultaneously binding to the plasma membrane, clathrin, cargo and cargo-specific adaptors (Collins et al. 2002; Ricotta et al. 2002; Cocucci et al. 2012). During nucleation, AP2 and clathrin recruit other adaptor proteins and proteins that bind plasma membrane, including FCH domain only (FCHO) proteins that additionally initiate membrane curvature. The cargo selection step involves recruitment of cargo to CCP through interaction with AP2 and/or additional cargo-specific adaptors. Clathrin triskelia that are recruited to AP2 polymerize to form pentagons and hexagons that build up the clathrin coat (coat assembly). Amphiphysin is another accessory protein that binds the plasma membrane, induces its curvature and recruits the GTPase dynamin to the neck of the forming vesicle. Dynamin polymerizes and through GTP hydrolysis, induces scission of the vesicle. Dynamin's role is well characterized for CME, but it is also implicated in other types of endocytosis (reviewed in Doherty and McMahon 2009; and in Mettlen et al. 2009). Uncoating is the last step of the cycle, and is initiated by synaptojanin, a phosphatase that converts PIP_2 to PI(4)P and releases adaptor proteins from the vesicle. Subsequent binding of auxilin, or cyclin G associated kinase (GAK), induces recruitment of ATPase heat shock cognate 70 (HSC70) that mediates disassembly of the clathrin coat. The components of the clathrin machinery are then ready for another CCV cycle. Invagination, scission and vesicle movement has been demonstrated to be supported by actin polymerization (reviewed in Yarar et al. 2005; Mooren et al. 2012) and the role of a motor protein, myosin 1E, has recently been indicated in this process (Cheng et al. 2012). The proteins that function in CME described above are only some of the key components of this process and other accessory proteins involved in CME have been described (Doherty and McMahon 2009; McMahon and Boucrot 2011).

Sorting signals and cargo-selective clathrin adaptors

Transmembrane cargos use a whole range of different unrelated sorting signals, including peptide motifs with or without posttranslational modifications, and the signal diversity prevents cargo competition and determines specificity of the uptake mechanism (reviewed in Traub 2009). AP2 is the core sorting adaptor and it recognizes the YXXØ motif (where Y is a tyrosine, X is any amino acid and Ø is a bulky hydrophobic amino acid) and an acidic di-leucine motif (Owen and Evans 1998; reviewed in Traub 2009). AP2 is usually accompanied by a number of monomeric cargo-specific adaptors, called clathrin-associated sorting proteins (CLASPs) that usually bind both AP2 and clathrin. Examples of CLASPs are the PTB domain-containing CLASPs (e.g. DAB2, ARH, NUMB), Ub selective CLASPs (epsin 1 and epidermal growth factor receptor substrate 15 (Eps15)), or β-arrestins (reviewed in Traub 2009). Eps15 and Epsin 1, CLASPs that participate in EGFR endocytosis, bind to Ub though their tandemly arrayed Ubiquitin-interacting motifs (UIMs) (Torrisi et al. 1999; Stang et al. 2004; Kazazic et al. 2009). They also bind to AP2, bind each other, and epsin 1 additionally interacts with clathrin (reviewed in Traub 2009). Such high interconnectivity between CLASPs and certain level of redundancy of sorting signals, can partly explain the fact that formation of CCPs and receptor internalization was observed to happen also upon depletion of AP2 (Motley et al. 2003; Johannessen et al. 2006; Maurer and Cooper 2006; Traub 2009). Multiple sorting signals can speed up cargo uptake, make it more robust and not dependent on a single CLASP (reviewed in Traub 2009). AP2 and CLASPs can themselves be positively and negatively regulated by posttranslational modifications and/or by binding proteins, like ubiquilin 2 (or PLIC2) that has been demonstrated to bind UIMs of Eps15 and epsin, and thus possibly outcompeting binding of ubiquitinated cargo (N'Diaye et al. 2008; Traub 2009).

Ubiquitination and its role in regulating ErbB protein levels

The UIMs that are present in Eps15 and epsin1 belong to the Ub-binding domain group of domains that all recognize Ub moieties, but with certain selectivity for the type of ubiquitination (reviewed in Dikic et al. 2009; Rahighi and Dikic 2012). Ub, a small protein consisting of 76 amino acids, can be covalently attached to a lysine (Lys) residue of another protein, by a chain of enzymatic reactions involving: Ub-activating enzyme (E1), Ub conjugating enzyme (E2), and Ub ligase (E3), a process called ubiquitination (reviewed in Hershko and Ciechanover 1998). Alternatively, Ub can be attached directly by the E2 enzyme (Hoeller et al. 2007). Ub itself contains seven internal Lys residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) to which another Ub can be attached. It has recently been demonstrated that Ub can also be conjugated through cysteine, serine or threonine residues by esterification (reviewed in Wang et al. 2012a) and the formation of linear head-to-tail-linked Ub chains (through the N-terminal methionine 1 residue of Ub) has been reported for NF-κB (Kirisako et al. 2006;

Tokunaga and Iwai 2012). The "Ub code" involves mono-, multimono- and polyubiquitination and polyUb chains can be homogenous or mixed, linear or branched, with conformation depending on the type of Lys-linkage. Due to this, ubiquitination mediates a number of various functions, with some functional redundancy (reviewed in Hicke 2001; Ikeda and Dikic 2008; Komander and Rape 2012; Schaefer et al. 2012). Functions for the Ub chains linked through Lys48 or though Lys63 have been well described. While Lys48-linked Ub chains mainly target proteins for proteasomal degradation, Lys63-linked Ub chains are involved in regulation of a number of cellular mechanisms, including endocytosis, lysosomal degradation, DNA damage response, protein translation, and cell signaling regulation (reviewed in Piper and Lehner 2011; Komander and Rape 2012). Lys11-linked Ub chains have been indicated to function in quality control by the pathway called endoplasmic reticulum (ER)-associated degradation (ERAD), in which misfolded proteins are targeted for proteasomal degradation (reviewed in Claessen et al. 2012). Ubiquitination plays an essential role in several steps of ErbB protein downregulation, which will be discussed in more details in the next section.

Multiple E3 ligases play important roles in both trafficking and controlling levels of ErbB proteins. There are three main groups of E3 ligases distinguished by possession of one of three E2-binding domains: the Really interesting new gene (RING) finger domain, the Homologous to E6-AP type (HECT) domain, or the U-box (reviewed in Pickart 2001). A RING finger domain containing Ub ligase, Casitas B-lineage lymphoma (Cbl), regulates degradation of EGFR and other components of the EGFR signaling complex and becomes degraded along with EGFR (Levkowitz et al. 1998; Levkowitz et al. 1999; Ettenberg et al. 2001). ErbB2 has been shown to bind Cbl, but the binding is not efficient enough to target ErbB2 for lysosomal degradation (Sorkin and Goh 2008; Carraway 2010). Degradation of ErbB2 has been reported to be regulated by other E3 ligases, namely, U-box-containing, Carboxyl terminus Hsc70-interacting protein (CHIP) and Cullin5-RING E3 ligase (Xu et al. 2002a; Zhou et al. 2003; Ehrlich et al. 2009). ErbB3 and ErbB4 cannot recruit Cbl (Levkowitz et al. 1996), but their steady-state levels have been demonstrated to be controlled by Neuregulin receptor degradation pathway protein 1 (Nrdp1) (Diamonti et al. 2002) and loss of Nrdp1 has been linked to oncogenesis (Yen et al. 2006). Levels of ErbB4 are also regulated, by the HECT family E3 ligases, including Nedd4 and Itch, that have been demonstrated to mediate degradation of ErbB4 (reviewed in Carraway 2010). The action of E3 ligases is counteracted by the action of deubiquitinating enzymes (DUBs). The function of DUBs is to reverse/prevent Ub-mediated processes (for example rescue proteins from lysosomal degradation), and to recycle Ub from cargo to the cytosol and thus maintain Ub homeostasis (reviewed in Clague and Urbe 2006; Millard and Wood 2006). UBPY, also known as USP8, and AMSH are endosome-localized DUBs that deubiquitinate EGFR and prevent its degradation (McCullough et al. 2004; Mizuno et al. 2005). UBPY (USP8) has also been reported to regulate endosomal trafficking of ErbB2 (Meijer and

van Leeuwen 2011) and POH1 is another DUB that has been reported to deubiquitinate ErbB2 (Liu et al. 2009). Furthermore, it has been suggested that DUBs together with E3 ligases can function in remodeling of Ub chains (Crosas et al. 2006). Components of the ubiquitination system are themselves regulated by ubiquitination and Ub-induced degradation (reviewed in Weissman et al. 2011). Cbl is targeted for degradation by autoubiquitination and by Nedd4- and Itch-mediated ubiquitination (reviewed in Ryan et al. 2006; and in Weissman et al. 2011). Cbl has additionally been demonstrated to be negatively regulated by a protein called T-cell ubiquitin ligand (TULA) that contains Ub-associated (UBA) and Src homology 3 (SH3) domains and, by binding to c-Cbl, outcompetes EGFR binding causing its reduced endocytosis and degradation (Feshchenko et al. 2004; Kowanetz et al. 2004). Later it was shown that overexpression of TULA inhibits also other dynamin-dependent endocytic pathways through sequestering dynamin (Bertelsen et al. 2007). Sprouty is another Cbl-interacting protein that when activated, competes with EGFR for binding to Cbl and thus inhibits EGF-induced ubiquitination and endocytosis of EGFR (Wong et al. 2001; Wong et al. 2002; Fong et al. 2003; Stang et al. 2004).

Endocytic transport of ErbB proteins

While endocytosis of ErbB2 and ErbB3 has been extensively studied only in the last decade, EGF-induced internalization of EGFR was one of the first ligand-induced endocytic pathways to be identified. Endocytosis of EGFR is by far the best characterized endocytic pathway for ErbB proteins, and serves as a model pathway also for other RTKs. Generally the half life of ErbB proteins is thought to correlate with their expression level, possibly due to saturability of the trafficking machinery. The EGFR turnover rate varies with a half life from 6-10 h in cells with moderate receptor level, up to 24 h or longer, in cells overexpressing EGFR. In unstimulated cells the EGFR is constitutively internalized and recycled, but the rate of this internalization does not exceed the general rate of the plasma membrane turnover. Addition of EGF, however, speeds up the turnover rate of EGFR strongly (reviewed in Sorkin and Goh 2008). The half life of ErbB2 is similar to that of unstimulated EGFR, ErbB4 has a reported half life of 5-7 h, while ErbB3 has the most rapid turnover, with a half life under 3 h (reviewed in Sorkin and Goh 2008).

Ligand-induced endocytosis and sorting of EGFR

The molecular mechanisms regulating EGFR endocytosis, such as alternative endocytic pathways, what adaptors that are involved, requirement of kinase activity, and different internalization signals including ubiquitination, are still debated. Already three decades ago it was demonstrated that EGFR was internalized from CCPs within minutes of ligand binding (Carpentier et al. 1982; Hanover et al. 1984).

CIE has been proposed to drive uptake of EGFR (Hinrichsen et al. 2003; Sigismund et al. 2005) and internalization of EGFR was postulated to depend on caveolin at high EGF concentrations (Sigismund et al. 2005). Later reports however, disclaimed involvement of caveolae in EGFR endocytosis both at high and low concentration of EGF (Kazazic et al. 2006; Sigismund et al. 2008; Rappoport and Simon 2009). Although some CIE pathways have been demonstrated for EGFR, CME is now generally believed to be the major pathway for the EGF-induced endocytosis of EGFR (reviewed in Sorkin and Goh 2008). The requirement of ubiquitination for EGFR endocytosis has also been discussed and several studies claimed that EGFR was internalized in absence of ubiquitination or in absence of Ub-binding adaptor proteins (reviewed in Sorkin and Goh 2008). Sigismund et al. postulated that low doses of EGF are not sufficient to induce ubiquitination of EGFR (Sigismund et al. 2005). Other groups have however demonstrated that EGFR is ubiquitinated also at low EGF doses (Kazazic et al. 2009; Sousa et al. 2012). EGFR ubiquitination happens already at the plasma membrane (Stang et al. 2000) and seems to depend on sustained EGFR kinase activity (Umebayashi et al. 2008). As revealed by mass-spectrometry analysis, within 5 minutes of stimulation with EGF, EGFR becomes multimono- and polyubiquitinated and the polyUb chains are primarily Lys63-linked (Huang et al. 2006; Umebayashi et al. 2008). Knock down of two isoforms of Cbl, c-Cbl and Cbl-b has been demonstrated to reduce internalization of EGF (Huang et al. 2006) and Cbl-mediated ubiquitination seems to be essential for translocation of EGFR to CCPs (Stang et al. 2004). Upon EGFR phosphorylation, Cbl binds to different phosphotyrosines on the C-terminal tail of EGFR either directly, or via growth factor receptor-bound protein 2 (Grb2) (reviewed in Sorkin and Goh 2008). Recruitment of Grb2 and Cbl was found to be both necessary and sufficient for EGFR endocytosis (Huang and Sorkin 2005). EGFR is the only ErbB protein that directly binds to AP2 (Sorkin and Carpenter 1993; Baulida et al. 1996) and requirement of AP2 for EGFR endocytosis has been reported (Rappoport and Simon 2009). However depletion of AP2 does not fully block EGFR internalization (Hinrichsen et al. 2003; Motley et al. 2003; Johannessen et al. 2006) and other adaptor proteins have been demonstrated to be involved in EGFR endocytosis. Epsin 1 and Eps15 have been postulated to mediate translocation of EGFR to CCPs, but while Eps15 localizes to the rims of CCPs, epsin 1 was found along the CCP (Kazazic et al. 2009; reviewed in Madshus and Stang 2009). Another Ub-binding protein, Cbl-interacting protein of 85 kDa (CIN85), was postulated to have a role in internalization of EGFR (Soubeyran et al. 2002; Schmidt et al. 2004), but recent studies demonstrate that it rather is involved in endosomal sorting for degradation and that its depletion promotes EGFR recycling (Schroeder et al. 2010; Ronning et al. 2011; Schroeder et al. 2012). It was recently reported that EGFR interacts with nonmuscle myosin (NM) II, a motor protein linked to actin filaments, and that this interaction is important for EGFR internalization and signaling (Kim et al. 2012). This further supports the involvement of the actin skeleton in EGFR endocytosis.

Once internalized, ubiquitinated EGFR is delivered to early/sorting endosomes from where it can be recycled back to plasma membrane or sorted towards the lysosomal degradation pathway. Ligand-bound and ubiquitinated EGFR is partially deubiquitinated after internalization. While TGF-α dissociation promotes deubiquitination and recycling of EGFR, EGF-EGFR complexes are reubiquitinated (Longva et al. 2002). c-Cbl and EGFR were then found to co-localize in early endosomes and the EGFR was further sorted to late endosomes and finally degraded (Longva et al. 2002; Umebayashi et al. 2008). This sustained ubiquitination mediates interaction of EGFR with several UIM-containing proteins of the endocytic machinery, as well as the sorting ESCRT machinery that is localized to the clathrin-containing microdomains on endosomes (reviewed in Raiborg and Stenmark 2009). EGFR is first recognized by the ESCRT 0 complex made of two subunits, namely hepatocyte-growth-factor-regulated tyrosine-kinase substrate (Hrs) and signal transducing adaptor molecule 2 (STAM2). Hrs binds ubiquitinated EGFR through its UIM, associates additionally with clathrin and PI 3-phosphate (PIP_3) enriched in the endosomal membrane (Raiborg et al. 2001; Raiborg et al. 2002; Raiborg and Stenmark 2009). STAM2 and Eps15 splice variant, Eps15b, additionally stabilize the sorting complex, through the Ub-UIM interactions (Bache et al. 2003b). Eps15b has been found to interact with Hrs and promote sorting of EGFR for degradation (Roxrud et al. 2008). ESCRT I complex is further recruited to early endosomal membranes and EGFR through interaction with Hrs (Bache et al. 2003a). Subsequently, ESCRT II and III complexes are recruited, EGFR is internalized into ILVs of MVBs, a process that requires deubiquitination (reviewed in Sorkin and Goh 2008; and in Raiborg and Stenmark 2009). Two deubiquitination enzymes, AMSH and UBPY (USP8), are known to modulate EGFR trafficking. AMSH interacts with both Hrs and STAM, is specific for the Lys63-linked Ub chains and has been proposed to rescue EGFR from lysosomal degradation, while UBPY (USP8) interacts with STAM, does not discriminate between Lys48- and Lys63-linked Ub chains and is important for sorting of EGFR to MVBs (reviewed in Clague and Urbe 2006; and in Madshus and Stang 2009). MVBs further fuse with lysosomes where EGFR becomes degraded. Alternatively receptors can be recycled back to plasma membrane through the tubular extensions in the limiting membrane of MVBs a process that also involves deubiquitination (reviewed in Sorkin and Goh 2008). While Eps15b directs EGFR for lysosomal degradation, another Eps15 isoform, Eps15S, has been reported to promote EGFR recycling (Chi et al. 2011). Additionally, proteasomes have been indicated to function in lysosomal sorting, even though EGFR itself does not seem to be degraded in proteasomes (reviewed in Madshus and Stang 2009). The EGFR trafficking model is summed up in Figure 6.

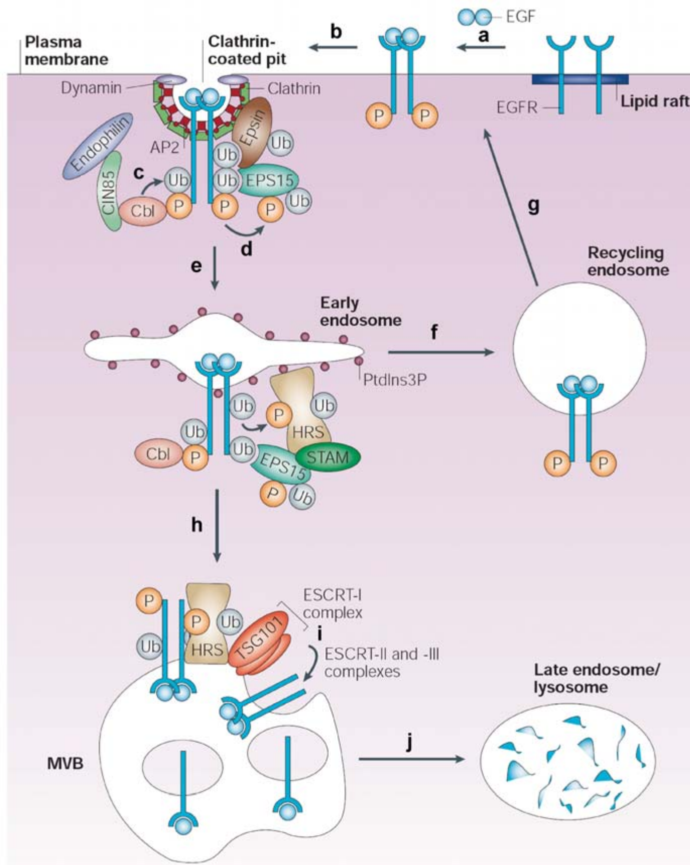


Figure 6. EGFR trafficking. a) EGF mediates EGFR phosphorylation and **b)** its translocation into CCPs. **c)** Cbl, which is associated with endocytic proteins, mediates EGFR ubiquitination. **d)** EGFR phosphorylates Eps15 and induces ubiquitination of Eps15 and Epsin. **e)** EGFR is then internalized into early endosomes, where it interacts with Hrs and ESCRT proteins. From there it can be either **f)** transferred to recycling endosome and **g)** recycled back to the plasma membrane, or **h)** stored on early endosomes where **i)** by the action of ESCRT machinery it is internalized into the ILVs. **j)** EGFR residing in MVBs is subsequently degraded in lysosomes. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Le Roy and Wrana 2005), © 2005. The figure legend is modified.

In addition to the classical ligand-induced CME, several other internalization mechanisms have been observed for EGFR. Activation of p38 MAPK has been reported to induce ligand-independent endocytosis of EGFR. A recent study showed that this endocytosis was clathrin-dependent, but that in contrast to the EGF-induced CME, did not require Grb2, but was instead AP2 dependent (Grandal et al. 2012). Moreover, at high expression levels, EGFR was demonstrated to be internalized by membrane ruffling/macropinocytosis (Chinkers et al. 1979; Haigler et al. 1979).

Endocytosis and sorting of ErbB2

Chimeric studies have demonstrated that ErbB2 does not associate with AP2 and undergoes weak endocytosis and degradation (Baulida et al. 1996). Its inefficient endocytosis was proposed to be due to the C-terminus of ErbB2 missing internalization signals or containing endocytosis-inhibitory signals (Sorkin et al. 1993), which could explain why an ErbB2 deletion mutant lacking C-terminal part appears to be unstable (Lerdrup et al. 2007). Austin et al. have proposed that ErbB2 is internalized and rapidly recycled (Austin et al. 2004). However, others found little or no ErbB2 in endosomal compartments (Longva et al. 2005; Lerdrup et al. 2006). ErbB2 has generally been accepted to be endocytosis inefficient, or even resistant, and one possible mechanism states exclusion of ErbB2 from CCPs and its association with plasma membrane protrusions (Hommelgaard et al. 2004). Moreover, overexpression of ErbB2 has been demonstrated to inhibit EGF-induced internalization and degradation of EGFR (Wang et al. 1999; Worthylake et al. 1999; Haslekas et al. 2005; Offterdinger and Bastiaens 2008). It was first suggested that this was due to rerouting of EGFR to the recycling pathway (Worthylake et al. 1999) but later studies demonstrated that EGFR-ErbB2 heterodimers were unable to induce formation of CCPs and that ErbB2 mediated retention of activated EGFR at the plasma membrane (Haslekas et al. 2005; Offterdinger and Bastiaens 2008; Hughes et al. 2009). ErbB2 is a client of the Heat shock protein 90 (Hsp90) that stabilizes ErbB2 through directly interaction (Xu et al. 2001; Xu et al. 2002b). Geldanamycin (GA) and its derivatives have been demonstrated to induce endocytosis of ErbB2 by inhibiting Hsp90 (Lerdrup et al. 2006; Pedersen et al. 2008). The GA-induced endocytosis is clathrin dependent and leads to lysosomal degradation, but the exact mechanism has been debated and both proteolytic fragmentation in the plasma membrane and internalization of intact ErbB2 have been observed (Tikhomirov and Carpenter 2000; Lerdrup et al. 2006; Lerdrup et al. 2007; Pedersen et al. 2008). Some studies reported that the GA-induced internalization of ErbB2 depends on proteasomes (Lerdrup et al. 2006; Lerdrup et al. 2007), while others stated that the proteasomal activity is not required for internalization of ErbB2 but for its lysosomal sorting (Pedersen et al. 2008). Moreover, GA treatment is known to induce ubiquitination of ErbB2 (Mimnaugh et al. 1996) and this ubiquitination has been linked to the action of the Ub ligases CHIP and Cullin5 (Xu et al. 2002a; Zhou et al. 2003; Ehrlich et al. 2009). In spite of the fact that ErbB2 has been shown to bind Cbl, the binding was not efficient enough to ubiquitinate and target ErbB2 for lysosomal degradation, but some oncogenic mutants of ErbB2 display increased, Cbl -dependent or Cbl-independent, ubiquitination and turnover (reviewed in Sorkin and Goh 2008; and in Carraway 2010). Additionally, the DUBs UBPY (USP8) and POH1 have been reported to regulate ErbB2 trafficking through deubiquitination (Liu et al. 2009; Meijer and van Leeuwen 2011).

Endocytosis and sorting of ErbB3 and ErbB4

Both ErbB3 and ErbB4, similarly to ErbB2, do not bind AP2 and were initially thought to be endocytosis-impaired (Baulida et al. 1996; Baulida and Carpenter 1997). ErbB3 was later proposed to undergo HRG-induced endocytosis, followed by recycling to the plasma membrane, due to the missing lysosomal-sorting signals in the C-terminal portion of ErbB3 (Waterman et al. 1998; Waterman et al. 1999). HRG exists in a number of isoforms (Breuleux 2007). Warren et al. demonstrated that the full length HRG, heregulin-extracellular domain (HRG-ECD), which in addition to the EGF like domain contains the N-terminal immunoglobulin-like domain, enhanced downregulation of surface-localized ErbB3, probably due to better ability to disrupt ErbB3 oligomers (Warren et al. 2006). The ubiquitin ligase Nrdp1 binds to the intracellular juxtamembrane region or to the kinase domain of ErbB3 (Bouyain and Leahy 2007), and previous studies have demonstrated that it controls ErbB3 steady-state by mediating its ligand-independent degradation (Diamonti et al. 2002; Qiu and Goldberg 2002). Later, Cao et al. proposed that HRG-stimulation leads to stabilization of UBPY (USP8) which in turn deubiquitinates and stabilizes Nrdp1, thus targeting ErbB3 for ligand-induced degradation (Wu et al. 2004; Cao et al. 2007). Nrdp1 was initially thought to target cell surface localized ErbB3 for proteasomal degradation (Qiu and Goldberg 2002), but Nrdp1-induced ErbB3 degradation has also been suggested to depend on lysosomes (Cao et al. 2007). Later it has been demonstrated that Nrdp1 preferentially associates with the nascent form of ErbB3 and mediates its degradation through the ERAD pathway, proposing a novel mechanism for ErbB quantity control (Fry et al. 2011). The nascent form of ErbB3 has also been reported to be stabilized by Hsp90 and its degradation was induced by GA (Gerbin and Landgraf 2010). ErbB4 steady-state has also been shown to depend on Nrdp1 (Diamonti et al. 2002). ErbB4 endocytosis and degradation is in addition regulated by the Nedd4 family of Ub ligases and the ErbB4 ubiquitination and endocytosis seems to depend on its isoform (Sundvall et al. 2008; reviewed in Carraway 2010). ErbB4 was proposed to be targeted for proteasomal degradation, but, like degradation of ErbB3, was also found to depend on lysosomes (Omerovic et al. 2007). ErbB4 can be cleaved and its soluble 80 kDa cytoplasmic portion (s80) translocates to nucleus where it can activate gene transcription and may act as nuclear chaperone for transcription factors (Omerovic et al. 2004; Williams et al. 2004). Constitutive nuclear localization of ErbB3 has also been postulated (Offterdinger et al. 2002; Koumakpayi et al. 2011) and the nuclear variant, ErbB3(80 kDa), has been reported to activate proliferative gene transcription (Andrique et al. 2012). Koumakpayi et al. suggested that ErbB3 nuclear localization depends on macropinocytosis, while CME might be involved in controlling cytoplasmic level of ErbB3 (Koumakpayi et al. 2011). Detailed information about the molecular mechanisms controlling ErbB3 and ErbB4 endocytosis and degradation still remain unclear.

ErbB proteins in cancer and anti-ErbB therapy

Hallmarks of cancer include sustained proliferation, evaded growth suppression, resistance to apoptosis, replicative immortality, induction of angiogenesis, invasiveness and metastasis, but as it recently became evident, also reprogramming of energy metabolism and evading immune response (reviewed in Hanahan and Weinberg 2011). Normal cells that successively accumulate mutations and acquire the mentioned hallmarks, can progressively enter the neoplastic state to become tumorigenic and eventually malignant (reviewed in Hanahan and Weinberg 2011). Cancer cells are also dependent on communication with the tumor microenvironment, including extracellular matrix and stromal non-cancerous cells, and para- and autocrine growth factor production allows for such short-range interchange (reviewed in Witsch et al. 2010). There are many phases of tumor growth that are controlled by growth factors, including clonal expansion, invasion, angiogenesis, as well as metastasis (reviewed in Witsch et al. 2010). The growth factor system is robust with multiple positive and negative feedback mechanisms and growth factors can be responsible for resistance and cell survival under radiotherapy and treatment with cytotoxic drugs (reviewed in Yarden 2011). ErbB proteins have been demonstrated to be involved in many types of cancers. There are multiple ways in which ErbB proteins contribute to oncogenesis: autocrine ErbB ligand production, ErbB upregulation due to gene amplification or defective degradation, subcellular relocalization, gain-of-function mutations, and dysregulation as well as cross-talk with other signaling pathways. Moreover, oncogenic activity of one ErbB member often depends on co-expression of another member of this family, or another RTK. They can also function as tumor biomarkers in cancer screening. Importantly, there is a broad spectrum of anticancer therapeutics that target ErbB proteins and many of those are already used in clinics.

ErbB proteins in cancer

EGFR is overexpressed in many cancer types including head and neck, ovarian, cervical, bladder, esophageal, gastric, lung, breast, endometrial and colorectal cancers and its overexpression often correlates with poor survival prognosis (reviewed in Nicholson et al. 2001). ErbB2 is overexpressed in a number of tumors (reviewed in Menard et al. 2003), but ErbB2 gene amplification is characteristic mostly for breast cancer and accounts for 20-30% breast cancer cases (reviewed in Tagliabue et al. 2010). Function of ErbB2 in driving proliferation of breast cancer cells has been shown to be dependent on ErbB3 (Holbro et al. 2003; Lee-Hoeflich et al. 2008; reviewed in Stern 2008; Vaught et al. 2012). Moreover, as mentioned before, overexpression of ErbB2 inhibits endocytosis and degradation of EGFR, and can thus contribute to sustained EGF-induced signaling (Haslekas et al. 2005). ErbB3 is

overexpressed in a number of cancers, including colorectal, ovarian, gastric, pancreatic, breast cancers and melanoma (Naidu et al. 1998; reviewed in Sithanandam and Anderson 2008; and in Campbell et al. 2010; Beji et al. 2011), and is linked to poor survival of cancer patients (Hirakawa et al. 2011). Correlation of ErbB3 overexpression with positive outcome in breast cancer patients has, however, also been demonstrated (reviewed in Koutras et al. 2010). Those opposing reports could depend on subcellular localization of ErbB3, since ErbB3 re-location to the plasma membrane has been linked to its increased activity and poor cancer prognosis (Sergina et al. 2007; Takikita et al. 2011), or on the presence of the secreted p85-soluble ErbB3, which was demonstrated to sequester HRG (Lee et al. 2001). Soluble forms of ErbB2 have also been observed and they can be generated by alternative splicing of *ErbB2* gene or by ADAM-mediated cleavage of the extracellular part of ErbB2 (reviewed in Tse et al. 2012). Such proteolytic cleavage creates a constitutively active truncated version of ErbB2 (p95HER2), and this is another way in which ErbB2 can contribute to oncogenesis (Christianson et al. 1998). Accordingly, ADAM proteins have frequently been found to be overexpressed in cancer (Lu et al. 2008). The role of ErbB4 in cancer has been debated and there are several reports about ErbB4 as a tumor suppressor, however, its overexpression in some cancer types correlates with metastasis and poor survival (reviewed in Carpenter 2003; and in Koutras et al. 2010). For instance, overexpressed ErbB4 seems to have an oncogenic activity in breast cancer (Abd El-Rehim et al. 2004) and its oncogenic activity depends on heterodimerization with ErbB2 (Mill et al. 2011). Because their frequent overexpression, ErbB proteins act as tumor biomarkers in tissues, but also in serum. Shedded extracellular part of ErbB2 has been proposed as a serum marker both for cancer and other non-cancer related diseases (reviewed in Zagozdzon et al. 2011; and in Tse et al. 2012). Secreted ErbB3 has been proposed as a serum marker for liver cancer (Hsieh et al. 2011) and elevated levels of ErbB4 ectodomain has been seen in serum of breast cancer patients (reviewed in Hollmen and Elenius 2010). ErbB3 mRNA levels have, in turn, been proposed as biomarker and treatment predictor for patients with ovarian cancer (reviewed in Amler 2010).

Somatic mutations have been observed for all ErbB proteins in cancers, and ErbB3 is the least represented member among them (reviewed in Rudloff and Samuels 2010). Mutations of EGFR have been well described and are localized to both the extracellular and intracellular domains (reviewed in Zandi et al. 2007; and in Pines et al. 2010). Extracellular domain mutation can disrupt the ligand binding, rendering receptor constitutive active, as is the case for the deletion mutant EGFRvIII that lacks domain I and parts of domain II and is present in several cancer types (reviewed in Zandi et al. 2007). EGFRvIII constitutively activates oncogenic signaling (Chu et al. 1997), escapes ubiquitination and lysosomal degradation, and is upon endocytosis instead recycled back to the plasma membrane (Grandal et al. 2007). EGFRvIV and EGFRV are intracellular deletion mutants that are found in glioblastomas

and they lack Grb2-mediated and/or direct Cbl-binding sites and do thus also escape degradation (reviewed in Roepstorff et al. 2008). Additionally, mutations in other plasma membrane proteins, alterations in the cytoskeleton, mutations resulting in loss of cell polarity and cell adhesion as well as mutations in components of the endocytic and recycling machinery can all affect the subcellular and lateral distribution of ErbB proteins and thus contribute to cancer (reviewed in Mosesson et al. 2008; Casaletto and McClatchey 2012). Perturbation in endocytic machinery can affect ErbB proteins directly, or indirectly by changing the cell architecture (reviewed in Casaletto and McClatchey 2012), and mutations, or loss of expression, of endocytic proteins, including clathrin heavy chain, endophilin II, Eps15, Cbl, Nrdp1, and subunits of ESCRT complexes, have been found in tumors (Yen et al. 2006; reviewed in Haglund et al. 2007; and in Mosesson et al. 2008).

Anti-ErbB therapeutics

Even though tumors harbor multiple mutations they are often addicted to one oncogene (Weinstein 2000). Targeted therapy is less toxic than chemotherapy and targeted therapy against ErbB proteins has proven to be an efficient treatment for a number of cancers. There are several strategies of anti-ErbB therapy and the drugs include tyrosine kinase inhibitors (TKIs), monoclonal antibodies (mAbs), and other agents, like ansamycin derivatives, which target Hsp90 and thereby its client proteins, including ErbB2 (Figure 7).

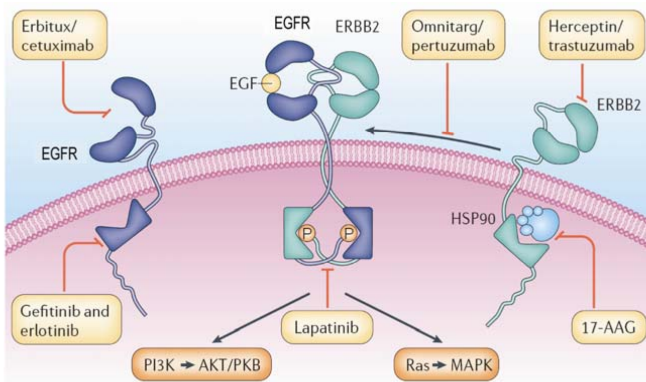


Figure 7. Anti-ErbB therapeutic agents. TKIs, like gefitinib, erlotinib, or lapatinib block the kinase activity of ErbB proteins. mAbs, like cetuximab, trastuzumab, or pertuzumab target ErbB proteins effecting their to resistance to ErbB TKIs and and among them are upregulation ion of ErbB3 at mRNA and ein level ADDIN EN.CITE ote><Cite><Author>Garrett</Aut Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Citri and Yarden 2006), © 2006. The figure legend is modified.

TKIs mimic ATP and prevent ErbB activation by binding to the ATP-binding pocket in the tyrosine kinase domain. They can bind either reversibly or irreversibly and are either specific or target more than one ErbB protein. Gefitinib (Iressa®) and erlotinib (Tarceva®) are examples of reversible TKIs specific for EGFR, while lapatinib (Tyverb®) is a reversible TKI which targets both EGFR and ErbB2. Both gefitinib and erlotinib prevent EGFR autophosphorylation and have an antiproliferative effect for EGFR-positive cancers, erlotinib does additionally lead to cell-cycle arrest and apoptosis (reviewed in Seshacharyulu et al. 2012). Lapatinib prevents phosphorylation and signaling of both EGFR and ErbB2 and has been shown to inhibit cell proliferation in ErbB2 overexpressing cancers (reviewed in Seshacharyulu et al. 2012).

Because of frequent overexpression of ErbB in tumors, mAbs have been designed to preferentially target malignant tissues. Such mAbs can bind to extracellular part of the specific receptor and can interfere with ligand binding and/or dimerization of receptors. Some antibodies also induce endocytic downregulation of the targeted receptor (reviewed in Friedlander et al. 2008). Additionally, most of the mAbs can induce the antibody-dependent cellular cytotoxisity (ADCC), a response which attracts immune cells, independently of other functional characteristic of the mAbs. Cetuximab (Erbitux®), is an anti-EGFR mAb approved for clinical use that binds the extracellular domain III (Li et al. 2005). It blocks ligand binding to EGFR, prevents EGFR from adopting its extended conformation, and thus blocks dimerization, induces EGFR endocytosis and degradation, blocks cell cycle and cell proliferation, and induces apoptosis and ADCC (reviewed in Friedlander et al. 2008). Cetuximab has been demonstrated to be efficient in combination with chemotherapy or radiotherapy (reviewed in Baselga 2001). Panitumumab (Vectibix™) and nimotuzumab (TheraCIM®) are other clinically approved mAbs that both block ligand binding but do not, or only partially mediate ADCC (reviewed in Friedlander et al. 2008). ErbB2 is also a target for mAbs: trastuzumab (4D5, Herceptin®), which is now approved for ErbB2-overexpressing breast cancer, and pertuzumab (2C4, Perjeta®) that is being tested in clinical trials (reviewed in Saxena and Dwivedi 2012). Trastuzumab binds to the extracellular domain IV of ErbB2 and has been observed to inhibit proliferation of breast cancer cells and xenografts, but the exact mechanism of its action is not fully understood (reviewed in Friedlander et al. 2008). Some of the studies reported that it induces internalization and downregulation of ErbB2, others argue that it induces recycling of ErbB2, while still others did not observe trastuzumab-induced internalization of ErbB2 (Longva et al. 2005; reviewed in Friedlander et al. 2008). Additionally, trastuzumab induces ADCC, inhibits angiogenesis and blocks ErbB2 ectodomain shedding (reviewed in Friedlander et al. 2008). In contrast to trastuzumab, pertuzumab has a better characterized mechanism of its antitumor action, and it is known that it binds the dimerization arm of ErbB2 thereby blocking ErbB2 dimerization with other receptors (Franklin et al. 2004). It also induces ADCC, affects glucose metabolism, but does not block

ErbB2 shedding (reviewed in Friedlander et al. 2008). Pertuzumab was demonstrated to inhibit ligand-induced formation of ErbB2-EGFR and ErbB2-ErbB3 heterodimers, and consequently signaling and cell growth (Agus et al. 2002; Takai et al. 2005; Sakai et al. 2007). It has recently been demonstrated that while trastuzumab disrupts preexisting ErbB2-ErbB3 heterodimers, pertuzumab instead inhibits HRG-induced ErbB2-ErbB3 heterodimers (Junttila et al. 2009). Prevented formation of ErbB2-EGFR by pertuzumab has also been shown to mediate EGF-induced internalization and degradation of EGFR in cells overexpressing ErbB2 (Hughes et al. 2009). There are several anti-ErbB3 mAbs that have been developed. Examples of those are the MM-888 mAb that causes growth inhibition, and MM-121 mAb that blocks ligand binding and activation of ErbB3. The bispecific mAb MM-111, which was designed to bind simultaneously to both ErbB2 and ErbB3, binds specifically to cells overexpressing ErbB2 and ErbB3 and blocks signaling and tumor growth (reviewed in Schoeberl et al. 2010; Jathal et al. 2011; McDonagh et al. 2012). In order to improve efficacy of the anti-ErbB mAbs, antibody conjugates have been developed, including trastuzumab-DM1, which is trastuzumab conjugated to a cytotoxic agent and is now in clinical trials for refractory breast cancer (reviewed in Stern 2012).

In addition to TKIs and mAbs, other drugs available for cancer treatment include Hsp90 inhibitors, like GA and its less toxic derivative 17-AAG (KOS-9539, Tanespimycin). Hsp90 inhibitors target ErbB2 for downregulation (Lerdrup et al. 2006). 17-AAG has proved promising in treatment of ErbB2-positive breast cancer and there are currently 17 different Hsp90 inhibitors that have entered clinical trials (reviewed in Neckers and Workman 2012).

Resistance to anti-ErbB therapy

Several of ErbB protein mutants have been demonstrated to be resistant to targeted anti-ErbB therapy. For instance, EGFRvIII is resistant to gefitinib and can also evade cetuximab treatment (reviewed in Wheeler et al. 2010). Often, cancers that initially respond to a drug, eventually escape from treatment. There are numerous mechanisms of such acquired resistance, including additional mutations, loss of tumor suppressors, overexpression of oncogenes (also ErbB proteins and their ligands), and compensatory ways to activate signaling pathways. For instance, TKI treatment has been demonstrated to cause secondary gain-of-function somatic mutations of EGFR (reviewed in Pines et al. 2010; and in Rudloff and Samuels 2010). TKI treatment was also demonstrated to lead to upregulation of ErbB3 at mRNA and/or protein level, or to cause relocation of ErbB2 to the plasma membrane (Sergina et al. 2007; Garrett et al. 2011; Grovdal et al. 2012). Another mechanism of resistance to gefitinib was shown to involve the HRG/ErbB3 pathway and correlated with strong overexpression of HRG and its sheddase

ADAM17 (reviewed in Sithanandam and Anderson 2008). In turn, loss of expression or inactivation of tumor suppressor lipid phosphatase, phosphatase and tensin homolog (PTEN), was linked to resistance to trastuzumab, lapatinib and gefitinib (reviewed in Wheeler et al. 2010; and in Stern 2012). Cross-talk between EGFR or ErbB3 with the upregulated c-Met receptor, or ligand independent activation of c-Met by constitutively active EGFRvIII, are other mechanisms that have been shown to drive resistance to TKIs (Arteaga 2007; Lai et al. 2009). Moreover, trastuzumab resistance has been correlated with signaling through EGFR, IGF-1R, c-Met and PC cell-derived growth factor (PCDGF) (reviewed in Tagliabue et al. 2010).

Combinatorial therapy is one potential strategy to overcome therapy resistance that has so far given promising effects. Combinations of trastuzumab with lapatinib, pertuzumab or 17-AAG or with inhibitors to other RTKs or downstream proteins have been investigated in clinics (reviewed in Tagliabue et al. 2010; and in Stern 2012). Combination of the anti-ErbB3 mAb MM-121 with anti-EGFR therapy, and combination of the bispecific anti-ErbB2/ErbB3 mAb MM-111 with anti-ErbB2 therapy, have shown promising results in the preclinical studies (Schoeberl et al. 2010; McDonagh et al. 2012). MM-121 appeared to overcome resistance to gefitinib and cetuximab (Schoeberl et al. 2010) and MM-111 seemed to increase efficiency of lapatinib and trastuzumab (McDonagh et al. 2012). In spite of the efficacy of the anti-ErbB therapy, most of the drugs cause severe side effects (reviewed in Force et al. 2007; and in Saxena and Dwivedi 2012). Moreover, the multiple mechanisms of resistance to each drug are not uniformly activated in all patients. Molecular screening of each tumor and deeper understanding of resistance mechanisms, would allow for the use of personalized drug cocktails. This in turn would increase therapy efficacy and minimize toxicity.

AIMS OF THE STUDY

The general objective of this thesis was to investigate molecular mechanisms involved in the endocytic downregulation of ErbB proteins with respect to clathrin-dependence, signals for endocytosis, and interactions between the members of the ErbB protein family.

The requirement of Cbl for endocytosis and subsequent degradation of EGFR has been well established. However, it has been debated to what extent internalization of EGFR depends on dimerization only or whether its kinase activity and possibly also ubiquitination is required. Studying these modifications in isolation has been a challenge, but our research group has have previously shown that ubiquitination is important for translocation of EGFR to CCPs and that epsin 1 could act as adaptor protein promoting translocation of ubiquitinated EGFR to CCPs. Those data suggest that ubiquitination is involved in the early steps of EGFR endocytosis. The aim of paper I was to investigate:

1. the role of Cbl in the early steps of EGFR endocytic transport, namely in the recruitment of activated receptor to CCPs and its subsequent internalization
2. the possibility that ubiquitination *per se* can act as a signal for EGFR endocytosis

ErbB3 overexpression and its heterodimerization with either ErbB2 or EGFR have been known to promote tumorigenesis. To what extent ErbB3 is internalized has been controversial and relatively little is known about the mechanisms that regulate downregulation of ErbB3. Previous studies from our group have demonstrated that overexpression of the endocytosis-resistant ErbB2 inhibits EGF-induced downregulation of EGFR due to ErbB2-EGFR heterodimerization, which could possibly contribute to sustained EGF-induced signaling. Furthermore, this inhibition was shown to be counteracted by incubation with pertuzumab. The aim of paper II was to study:

3. the mechanism of ErbB3 internalization and whether, as in case of EGFR, receptor phosphorylation is a signal for internalization and subsequent degradation of ErbB3
4. the possible impact of EGFR-ErbB3 heterodimerization on endocytosis of EGFR

The aim of paper III was to investigate:

5. the potential inhibitory effect of ErbB2 on ErbB3 endocytosis and degradation
6. the effect of pertuzumab on HRG-induced signaling and downregulation of ErbB3 in cells co-expressing ErbB2

SUMMARY OF PAPERS

Paper I

A chimeric pre-ubiquitinated EGF Receptor is constitutively endocytosed in a clathrin-dependent, but kinase-independent manner. *Vibeke Bertelsen, Malgorzata Magdalena Sak, Kamilla Breen, Marianne S. Rødland, Lene E. Johannessen, Linton M. Traub, Espen Stang, and Inger Helene Madshus*

In this paper we studied the role of Cbl and ubiquitination in the early steps of EGFR endocytosis. Using two different pools of small interfering RNA (siRNA) to Cbl, we first confirmed that knock-down of c-Cbl and Cbl-b inhibited ubiquitination of EGFR and internalization of ^{125}I-EGF. Our immuno-EM data, demonstrated that upon knock down of c-Cbl and Cbl-b, EGFR localized mainly to the rim, and not to interior part of the coated pits, indicating that Cbl is needed for translocation of the activated EGFR into clathrin coated pits. To further investigate whether ubiquitination could act as a signal for endocytosis of EGFR, we created a chimeric protein, EGFR-Ub_4, by fusing a tandem of 4 ubiquitins via a linker to the C-terminal tail of EGFR. As observed by confocal microscopy and biotinylation experiments, this EGFR-Ub_4 chimera, in contrast to the wild type EGFR, was internalized from the plasma membrane and localized to early endosomes in the absence of added ligand. Our co-immunoprecipitation experiments showed constitutive interaction of EGFR-Ub_4 with epsin 1 and Eps15. Moreover, overexpression of truncated mutants of epsin 1 or Eps15, containing their UIMs, efficiently inhibited the constitutive internalization of EGFR-Ub_4. Using siRNA to clathrin, we demonstrated that, just like wild type EGFR, EGFR-Ub_4 was internalized in a clathrin-dependent manner. However, in case of EGFR-Ub_4, this internalization occurred also upon inhibition of EGFR kinase activity and without interaction with c-Cbl. Thus, based on our data, we concluded that the covalent addition of 4 ubiquitins in tandem overrides the requirement of phosphorylation for the clathrin-dependent internalization of EGFR. Our results support the notion that Cbl-mediated ubiquitination, not only is involved in the endocytosis of EGFR, but it can in itself be a signal for endocytosis of EGFR from the clathrin coated pits.

Paper II

The oncoprotein ErbB3 is endocytosed in the absence of added ligand in a clathrin-dependent manner. *Malgorzata Magdalena Sak*, Kamilla Breen*, Sissel Beate Rønning, Nina Marie Pedersen, Vibeke Bertelsen, Espen Stang, and Inger Helene Madshus*

*These authors contributed equally to this work

In this paper we investigated activation and endocytic downregulation of ErbB3 in cells expressing ErbB3 alone or co-expressing ErbB3 and EGFR. Using confocal microscopy and flow cytometry, we initially observed that, in contrast to EGFR and ErbB2, ErbB3 in the absence of added ligand was downregulated from the plasma membrane, internalized to intracellular vesicles and partly localized to early endosomes. ErbB3 endocytosis was independent of other ErbB proteins, and we demonstrated that it was markedly reduced upon siRNA-mediated depletion of clathrin. We then compared degradation of ErbB3 with and without the ligand. Consistently with its constitutive internalization, ErbB3 was degraded in absence of added ligand. ErbB3 degradation was, however, slightly enhanced by incubation with HRG-ECD, which also previously has been shown to accelerate turnover of ErbB3. In line with recent reports, our Western blotting experiments confirmed that ErbB3 had a marginal kinase activity, but that it was dependent on ligand-induced heterodimerization for efficient phosphorylation and activation of downstream signaling. Furthermore, because ErbB3 appeared to be phosphorylated to some degree without added ligand, we treated cells with kinase inhibitors and observed that ErbB3 was internalized even upon inhibition of its kinase activity and also in the presence of a c-Met kinase inhibitor. By examining activation of downstream signaling, we observed that functional EGFR-ErbB3 heterodimers were formed upon incubation with ligand to ErbB3. Moreover, HRG-induced heterodimerization of ErbB3 with EGFR appeared to reduce internalization of ^{125}I-EGF. Altogether our data demonstrated that ErbB3 is internalized in absence of added ligand and in a clathrin dependent manner. Based on our results we also conclude that dimerization of ErbB3 with EGFR negatively impacts on EGF-induced endocytosis of EGFR, which could possibly contribute to prolonging of EGF-induced signaling.

Paper III

Pertuzumab counteracts the inhibitory effect of ErbB2 on degradation of ErbB3. *Malgorzata Magdalena Sak, Monika Szymanska, Vibeke Bertelsen, Max Hasmann, Espen Stang, and Inger Helene Madshus*

In this paper we studied the effect of ErbB2 on endocytic downregulation of ErbB3 and HRG-induced signaling, and the effect of pertuzumab on these processes in cells co-expressing ErbB2 and ErbB3. We initially examined HRG-induced signaling in cell lines expressing ErbB3 alone, or in combination with ErbB2 or EGFR or both. Our results confirmed that ErbB3 heterodimerization was required to efficiently phosphorylate ErbB3 and activate downstream signaling pathways. We then investigated endocytosis of ErbB3 in cells co-expressing ErbB2 by flow cytometry and confocal microscopy analyses and we observed that ErbB2 did not block internalization of ErbB3 in absence of added ligand. We further compared degradation rate of ErbB3 in cell lines that overexpress ErbB2 and cell lines that have no or little ErbB2. Our Western blot experiments demonstrated that HRG-induced degradation of ErbB3 was significantly slowed down in cells expressing high levels of ErbB2 and that we were able to counteract this effect by incubating cells with pertuzumab. These data indicate that formation of ErbB2-ErbB3 heterodimers inhibits downregulation of ErbB3 and supports the notion that pertuzumab inhibits ErbB2 dimerization. We additionally confirmed the inhibitory effect of pertuzumab on ErbB2-ErbB3 heterodimerization, by demonstrating that pertuzumab generally inhibited ErbB3 phosphorylation in cells expressing ErbB2 and efficiently reduced HRG-induced downstream signaling in cells expressing low levels of ErbB2.

METHODOLOGICAL CONSIDERATIONS

This section provides discussion of a selected set of methods used in this thesis, their general aspects, advantages and limitations important for data interpretation. Detailed technical description of the methods is provided in the attached papers. All presented experiments have been done at least three times, unless otherwise stated. Statistical analysis has been used to assess significance.

Experimental model

Choice of cell lines and stable transfection

The use of cultured immortalized cells as a model system allows for performing and replicating all experiments, in a more or less similar setting. In our studies we used following human cell lines. HeLa cells were used to study EGFR (paper I), because of their moderate EGFR expression level, because they are easy to transfect and have previously been used for similar studies. Since the HeLa cells, that we used, did not express detectable amounts of ErbB3, MCF-7 cells and SK-BR-3 cells were used to study endogenous ErbB3 (paper II and III). These two cell lines express relatively equal amounts of ErbB3, but vary with respect to ErbB2 expression, which was an advantage when studying the effect of ErbB2 on ErbB3 in paper III.

Porcine aortic endothelial (PAE) cells were used for most experiments. PAE cells do not express ErbB proteins endogenously. Creation of stable cell lines, expressing different ErbB proteins alone or in combination, made it possible to study each ErbB protein in isolation, and to study the ways they influence each other. In paper II, we studied ErbB3 kinase activity independently of other ErbB proteins using PAE cells expressing ErbB3 only. The PAE cell system also allows for the observation of mutant/modified ErbB proteins, without the interference from wild type ErbB proteins. However, expression of a protein in its unnatural environment (such as cells of another species) is to some extent an artificial situation. The PAE.EGFR cell line has previously been well characterized (Carter and Sorkin 1998). However, because PAE cells, as compared to human cells, may have different control mechanisms, we performed a number of controls, and compared ErbB protein localization, activation and downregulation in PAE cells with other established cell lines. As described in the various papers, we found no unusual behavior of ErbBs expressed in PAE cells. PAE cells are relatively easy to transfect, but their physiology represents both advantages and disadvantages. The cells are flat and

widespread which, when observed in a microscope, gives rise to a large cytoplasm to nucleus ratio. This makes it easy to visualize cellular compartments under the microscope. However, at the same time it is difficult to visualize the clear plasma membrane staining along the edge of the cell, as one often additionally captures fluorescence from the apical or the basal plasma membrane.

All cells were kept in culture at < 100 % confluence and for a limited number of passages to minimize the number of mutations and to limit the loss of stably expressed ErbB protein. Homo/heterogenicity of the cell population, with respect to expression of the actual ErbB protein, was for each cell line regularly monitored using flow cytometry. Highly heterogeneous cell populations were excluded. Western blotting was performed to analyze the total receptor level. Cells were regularly tested to exclude mycoplasma infections.

Stimulation with ligands

We have not tested our cells for the production of autocrine ligand(s). For that reason, whenever describing receptor internalization/activation, when using the expression “constitutive” we actually mean “in absence of added ligand”. In order to avoid the interference of potential external ligands in experiments where we studied receptor phosphorylation or ubiquitination, the cells were incubated with serum free media for at least 30 min prior to addition of ligand. We used different concentrations of EGF for different methods. 1 ng/ml ^{125}I-EGF was used for internalization studies, because it has been reported that higher concentrations may induce clathrin-independent endocytic pathways (Sigismund et al. 2005; Roepstorff et al. 2008). 15 ng/ml was used in confocal microscopy analysis to ensure visualization of the internalized EGFR. 60 ng/ml (10nM) EGF was used for Western blot experiments to obtain clearly detectable phosphorylation and ubiquitination of EGFR. For stimulation with HRG we used 10 nM (80 ng/ml HRG β1 and 269 ng/ml HRG β1 ECD) to ensure efficient activation of ErbB3.

Transient protein expression

Transient transfection with plasmid is a way of inducing or enriching the expression a protein of choice. Transient transfection often results in protein overexpression, which can be either an advantage or a disadvantage. Transient transfection can be used to raise the protein expression level to detectable levels, like when expressing ErbB3 in HeLa cells (supplementary information in paper II). Overexpression of a dominant mutant protein can out-compete the endogenous wild type protein and thus allow studies both of protein interactions, and the function of a protein or its particular domains,

like in case of Eps15 and epsin 1 mutants in paper I. Overexpression of a protein can however cause protein aggregation and/or altered protein localization which can result in physiologically irrelevant output, like change in localization, trafficking, and/or half life. Saturation of the molecular machinery responsible for those processes can be one of several possible reasons. Frequent low transfection efficiency is another problem. Firstly, if only small fraction of cells express the protein of interest, the effect of its expression might be too weak to be detected. Secondly, transient transfection often results in a cell population where the expression of the transfected protein is highly variable form cell to cell, and this may affect the read out in assays that look at cell population a whole (e. g. Western blots and internalization assays using ^{125}I-labeled ligands). In that respect, the use of stably transfected cell lines, with moderate and more uniform protein expression is preferable, although creation of stably transfected cell lines may be more time consuming. The non-uniform expression of a protein can, however, also be advantageous. In assays, like confocal/fluorescent microscopy, when looking at single cells, transfected and non-transfected cells can be compared in one image and function as internal controls, like in case of Eps15 and epsin 1 mutants in paper I.

Protein knock-down by RNA interference

RNA interference first discovered in plants (Hamilton and Baulcombe 1999) has since widely been used in mammalian cells as a tool for post-translational suppression of gene expression. Even though it is considered a highly specific method, because it is based on the RNA sequence complementarity, siRNAs often unspecifically recognize similar sequences (so called off-target effect). The use of a pool of different siRNA sequences against one protein may improve the knock-down efficiency, but may also increase the chance for off-target effects, and can result in data misinterpretation. Nevertheless, whether one uses a single siRNA sequence or a sequence pool, especially for sequences not tested before, one should verify results with an at least one different siRNA sequence (or sequence pool) targeting the same protein. This was done for the Cbl knock down in paper I, where two siRNA pools were used. Moreover, it has been reported that siRNA can induce sequence independent suppression of protein synthesis, enhancement of mRNA degradation, and secretion of cytokines, through the Toll-like receptor 3 and induction of type I interferon (Kariko et al. 2004a; Kariko et al. 2004b), which makes it another pitfall of this method. The use of liposome based transfection can itself affect cellular functions. To somewhat correct for unspecific effects of the siRNA and/or transfection reagent, we included transfection with control siRNA in our experiments. Control siRNA (like commercially available negative control sequence in paper I and II) does not have any target or it targets a protein that is not expressed in cells used (green fluorescent protein-specific sequence in paper I and II). Concentration of

each siRNA sequence was optimized separately and we used the lowest concentration of siRNA that efficiently knocked down protein expression, in order to minimize cell toxicity. Cellular processes and pathways are complex and involve proteins with redundant function. Therefore it is important to keep in mind that depleting one protein one can potentially activate an alternative “rescue” protein/pathway that would normally not exist under physiological conditions. Alternatively, the observed effect of protein knock down might be an indirect effect, where protein depletion affected availability of other proteins that belong to the same protein complex or/and cellular process (e. g. endocytic pathway).

Biochemical methods

Antibody specificity

Immunological methods are based on antigen epitope-antibody interaction and their reliability depends on specificity of this interaction. Immunological recognition depends in addition to antibody specificity, on a number of factors including, protein state (native/denatured), antibody concentration, incubation and blocking solutions, incubation time and temperature. Those factors usually vary for different immunological methods. For commercially available antibodies (which is the case for all antibodies included in the thesis), manufacturer usually provides instructions on how the antibody should be used for selected methods. We nevertheless verified specificity and optimized concentration and specificity of each antibody separately for each method and cell line, when possible. PAE cells expressing single ErbB proteins are especially useful for testing specificity of anti-ErbB and anti-phospho-ErbB antibodies.

Kinase inhibitor specificity

Due to high sequence similarity among ErbB proteins, and often also other RTKs, kinase inhibitors frequently target kinase domains of related proteins within a protein family. Just like in case of testing antibody specificity, PAE cells are a useful tool for testing specificity of the kinase inhibitors. In paper II, using PAE.ErbB3 expressing ErbB3 and no other ErbB proteins, we were able to demonstrate that the kinase inhibitor AG1478, previously shown to target the EGFR and ErbB2 kinase domains (Shushan et al. 2004; Yang et al. 2007), did also block the ErbB3 kinase activity. The EGFR kinase inhibitor PD153035 did as well block the ErbB3 kinase activity (data not shown), however this had little consequence for our experiments in paper I, since we only used cells that expressed EGFR or EGFR-Ub_4, and no other ErbB proteins. The c-Met kinase inhibitor SU11274, used in paper II to block the c-

Met kinase in PAE.ErbB3 cells, was tested and appeared not to affect HRG- and EGF-induced signaling (data not shown).

Western blot, Immunoprecipitation (IP), Co-IP, Biotinylation

Western blot was used to assess protein presence and its relative expression level in cells. In this method, antibodies recognize proteins in their denatured state. Proteins are separated based on their molecular weight. By comparing analyzed protein samples to a molecular weight marker, one can usually exclude unspecific protein "bands" from analysis. The method is sensitive enough to distinguish EGFR (170 kDa) from ErbB2 (185 kDa), in case of unspecific antibody binding (in paper III). In addition to looking at the molecular weight, we, whenever possible, tested antibody specificity by including control samples from cells with no expression, or overexpression of the targeted protein. Using Western blot, one cannot directly compare the expression levels of different proteins, because each antibody bind to their respective targets with different efficiency. We could, however, compare the relative expression level of a single protein between samples from different conditions/cell lines, using the same specific antibody. To visualize proteins, horseradish peroxidase (HRP)-produced chemiluminescence was recorded as an image on the computer screen and pixel intensity was quantitated using special software. Such densitometric quantitation is not very accurate. It can be affected by background noise, band intensity, size and position of the manually set region of interest (ROI). To correct for variance between the samples loaded on the gel, we normalized values to the values from simultaneously loaded control-proteins. When developing Western blot, we were careful not to over-expose the membrane, because once over-exposed, the intensity of the oversaturated signal is no longer proportional to the level of protein. Due to this inaccuracy, standard deviations from repeated experiments often do not confirm significance. For most our experiments we therefore presented densitometric quantitation of a representative Western blot, and the Western blot image that was quantitated was always presented along with the quantitation. Images of Western blots were processed using Adobe Photoshop CS4, to make sure that the final image remained representative adjustments were applied to the whole image not to single bands within the picture.

There is a limited volume of protein solution one can analyze at a time by Western blotting. IP method was used to enrich a protein of interest in a sample, or to exclude other proteins from the sample. This is important when analyzing phosphorylation and ubiquitination. In IP, proteins are precipitated from the cell lysate by antibodies coupled to protein A or G (commercially pre-coupled to magnetic beads). Such precipitates are then eluted and analyzed by Western blotting. Posttranslational modifications such as

phosphorylation and ubiquitination are not affected by SDS-induced protein denaturation but their removal can still occur during preparation. To avoid this we, in addition to protease inhibitor cocktail used for Western blot, supplied the lysis buffer with inhibitors of phosphatases and DUBs to avoid degradation of those modifications. Control samples with protein A/G-coupled beads, without the antibody, were used as control for unspecific binding of proteins to the beads. When protein complexes were analyzed by co-IP experiments, no SDS was used under precipitation to prevent dissociation of protein complexes. SDS was, however, included in elution of the precipitate and Western blotting was preformed as usual.

Biotinylation of surface proteins was used to study internalization of EGFR (paper I). Biotinylated proteins were first allowed to enter the cells, and then residual biotin was stripped from the surface of cells. After cell lysis, biotinylated proteins were precipitated using streptavidin-coupled magnetic beads. Stripping efficiency was estimated by control samples from cells incubated on ice only, where ideally no internalization should have occurred.

Immunofluorescence, flow cytometry and confocal microscopy

Immunofluorescence was used as a tool to study fixed cells both by flow cytometry and confocal microscopy analyzes. Confocal microscopy served as a qualitative method to analyze subcellular localization of, and co-localization between proteins in cells, using fluorochrome-conjugated ligands or secondary antibodies. Confocal microscopy was also used to assess endocytic ability of the ErbB proteins. First, the primary antibody was bound on ice, and after washing off the residual unbound antibodies, the bound antibodies were allowed to internalize by incubating the cells at 37 °C for a period of time. After fixation and permeabilization of cells, the antibody was localized using fluorochrome-conjugated secondary antibody. The anti-ErbB3 antibody was first tested by flow cytometry to exclude its ability to induce endocytosis of ErbB3 (paper II). In each confocal microscopy experiment many cells were analyzed to correct for individual cell variations. We used ammonium chloride to quench fluorescent background coming from the fixation reagent. Due to the use of relatively robust fluorochromes, bleaching did not seem to be a problem in our experiments. One of the advantages of fluorescent/confocal microscopy is that by using different fluorochromes, one can simultaneously analyze the localization of multiple antigens in the same sample. Such multiple staining, however, increases the chance for cross-reaction. We therefore tested both primary and secondary antibodies to ensure minimal unspecific binding and exclude cross-reaction with other antigens/antibodies. Bleed-through, caused by overlapping excitation and/or emission spectra of the used fluorochromes, is another

problem when using multiple fluorochromes. To avoid that, the images of different fluorochrome were taken sequentially, starting from the dye with the longest wavelength peak emission and finishing with the shortest. To reduce background fluorescence, the average of 3-4 images was used for the analysis and the Kalman filter was applied for the images taken with the Olympus microscope. Images were processed using Adobe Photoshop CS4, the adjustments were applied to the whole image in a way that the final image remained representative. When talking about co-localization of proteins, it is important to keep in mind that the images were only studied in the x and y plane. Even though the thickness of the optical section (Z plan), depending on the pinhole size, was relatively thin, we cannot be certain that what appeared as co-localized structures were co-localized also in the z plane.

Flow cytometry was used to study downregulation of ErbB proteins from the plasma membrane by measuring cell surface protein in a population of non-permeabilized cells. One of the advantages of this method is its quantitative nature and the possibility for analysis of a large number of cells in a short period of time. One can also exclude dead cells from analysis. Median value of the signal distribution was used to compare samples. Average of parallels from multiple experiments was calculated with pooled standard deviations and p value was calculated to estimate statistical significance. Primary and secondary antibodies were tested to exclude cross-reaction with other antigens. Antibody concentrations were optimized to obtain antigen saturation, in order to avoid signal variation caused by variation in pipeting, which is especially important for quantitative methods. Control samples, incubated only with the fluorochrome-conjugated secondary antibodies, were included in the analysis to visualize the background signal.

Immuno- electron microscopy (Immuno-EM)

Immuno-EM has a higher resolution than the fluorescent/confocal microscope and served as a tool to study subcellular localization of proteins, including localization within particular cellular compartments/organelles. EGFR localization to clathrin-coated pits was studied in paper I. Another advantage of this method is that morphology of the subcellular membranous structures can often be recognized without special markers, due to the electron dense protein coats. Quantitations in paper I were done by counting the total number gold particles localized to the plasma membrane of a minimum of 20 randomly chosen cells. As electron microscopy allows clathrin coated pits to be identified by their characteristic electron dense coat, the counted gold particles were grouped with respect to being localized outside or within coated pits.

Internalization of radio-labeled ligand

Internalization of ^{125}I-EGF served as a quantitative method to study the ligand-induced internalization ratio of EGFR. High detection sensitivity of this method allowed for use of low concentration of radiolabeled EGF. Unfortunately, iodinated HRG was not commercially available, and because it has been reported that iodinated HRG loses its high binding affinity possibly due to sulfoxidation of methionine residues (van der Woning and van Zoelen 2009), we did not include iodinated HRG in our studies.

GENERAL DISCUSSION

Detailed discussion of the results is provided in each paper. This section presents discussion of the main findings, with respect to the aims of the study and in the context of recent findings and often contradictory literature.

Ubiquitination of EGFR as a signal for endocytosis

A general difference between the constitutive and the ligand-induced endocytosis is that the latter requires switching on/exposure of the endocytic signal that is otherwise absent in the unstimulated protein. Post-translational modifications are such dynamic signals. Both phosphorylation and ubiquitination have been proposed to be necessary for endocytic downregulation of EGFR. The requirement of Cbl for EGFR degradation has been well established, however, the exact role of Cbl in initial steps of EGFR internalization has been debated. It has been demonstrated that not only the protein adaptor function, but also the Ub-ligase function of Cbl is essential for EGFR internalization (Huang and Sorkin 2005; Pai et al. 2006). It has however been unclear whether it is Cbl-mediated ubiquitination of EGFR, or rather ubiquitination of accessory endocytic proteins, that is required for EGFR endocytosis. Results from our group have demonstrated that Ub and the Ub-binding adaptor protein epsin 1 are involved in translocation of EGFR to CCPs, which indicated the importance of EGFR ubiquitination in early steps of its endocytosis (Stang et al. 2000; Stang et al. 2004; Kazazic et al. 2009). In paper I, we further strengthened this hypothesis by showing that expression of Cbl was required for effective translocation to CCPs and subsequent internalization of EGFR.

Sigismund et al. postulated that at low doses of EGF, the non-ubiquitinated EGFR is internalized in a clathrin-dependent manner, but when exposed to higher doses of EGF, EGFR phosphorylation is strong enough to induce EGFR ubiquitination and promote EGFR internalization by a non-clathrin pathway (Sigismund et al. 2005). Our data, however, show for the same cell line (HeLa cells) that EGFR is ubiquitinated already when stimulated with 1 ng/ml EGF (Kazazic et al. 2009), and a recent study has demonstrated EGFR ubiquitination upon exposure to 1.5 ng/ml EGF in dynamin depleted fibroblasts (Sousa et al. 2012). The fact that Sigismund et al. did not see the ubiquitination under such conditions could indicate insensitivity of the method used, or differences between HeLa cell clones. Ubiquitination seems to be a well conserved internalization signal, several studied have reported its requirement for internalization of surface cargo in both yeast and mammals (Galan et al. 1996; Hicke and Riezman 1996; Geetha et al. 2005; Belouzard and Rouille 2006; Miranda et al. 2007; Erpapazoglou et al. 2008).

Our results in paper I demonstrate that a covalently linked chain of 4 Ubs was sufficient to induce internalization of EGFR. Chimera-EGFR fused to a monoubiquitin has been studied by several other groups (Haglund et al. 2003; Mosesson et al. 2003; Sigismund et al. 2005) and internalization of monoubiquitinated EGFR was proposed to depend on caveolin, not on clathrin (Sigismund et al. 2005). The truncated EGFR used in that study, however, lacked its intracellular domain and could therefore possibly be internalized by other mechanism that the wild type. It has additionally been demonstrated that the EGFR upon activation is multimono- and polyubiquitinated and that the Ub chains are mainly Lys63-linked (Huang et al. 2006). In most cases monoUb seems to be insufficient to mediate internalization of cargo and increasing length of the fused tandem-Ub was demonstrated to accelerate the clathrin-dependent internalization and improve association of cargo with Eps15 and epsin 1 (Barriere et al. 2006; Ma et al. 2012). Moreover, epsin 1 and Eps15 preferentially bind to polyUb chains (Barriere et al. 2006; Hawryluk et al. 2006). When studying the function of Ub by using tandemly fused Ubs, one has to be aware of the specificity of different UIMs for binding to different polyUb chains (Sato et al. 2009; Sims and Cohen 2009). Conformation of the Lys63-linked Ub chain, more than conformation of the Lys48-linked Ub chain, resembles the linear Ub chain. However some Ub binding proteins are known to discriminate also between linear and Lys63-linked Ub chains (Komander et al. 2009; reviewed in Rahighi and Dikic 2012). This did not seem to be a problem in our studies, since we observed constitutive association of EGFR-Ub_4 with epsin 1 and Eps15 and expression of the UIM containing truncated mutants of these adaptors sequestered EGFR-Ub_4 at the plasma membrane. It has been demonstrated that a 15KR EGFR mutant, with 15 Lys residues in its kinase domain mutated to arginine, displayed 99% reduction in ubiquitination but was internalized at a similar rate as the wild type receptor (Huang et al. 2007). Based on that study it was claimed that ubiquitination of EGFR itself is not required for its internalization. It is however possible that the 1% residual ubiquitination of the 15KR mutant could be sufficient to induce its endocytosis. The fact that in our study, the tetraUb chain was sufficient to induce the constitutive association of EGFR with epsin 1 and Eps15 and subsequent clathrin-dependent internalization of EGFR, suggests that EGFR does not need to be heavily ubiquitinated in order to be internalized. It has been difficult to separately study the contribution of kinase activity and ubiquitination to endocytosis of EGFR (Huang and Sorkin 2005; Huang et al. 2007). Wang et al. proposed that EGFR is internalized independent of kinase activity and that its internalization is controlled by receptor dimerization (Wang et al. 2005; Wang et al. 2007). However, several groups, in addition to our data, postulated that ligand-induced receptor dimerization and its subsequent autophosphorylation is needed for EGFR internalization (reviewed in Madshus and Stang 2009). Upon EGF stimulation, Grb2 binds to phosphorylated EGFR and recruits Cbl and it has been demonstrated this recruitment is required and sufficient to induce CME of EGFR (Huang and Sorkin 2005). In paper I,

by blocking kinase activity of EGFR-Ub_4, we were able to demonstrate that ubiquitination *per se* can act as internalization signal and thus the main role of kinase activity seems to be recruitment of Cbl. We do not however exclude possibility that under physiological conditions, several mechanisms may act in parallel to mediate EGFR endocytosis. Indeed, a recent publication proposed four redundant, or partially cooperative, mechanisms controlling the clathrin-dependent endocytosis of EGFR, namely ubiquitination, AP2 binding, and Grb2 binding, and acetylation of C-terminal Lys residues (Goh et al. 2010). Those mechanisms may vary between cell lines and depend on experimental setting and can be alternatively utilized in physiological conditions.

Endocytosis of ErbB3

ErbB3 has been postulated to be inefficiently endocytosed. Firstly, it did not associate with AP-2 and did not display ligand-induced decrease in half life (Baulida et al. 1996). Secondly, ErbB3 was observed to have slow rate of HRG-induced internalization (Baulida and Carpenter 1997). In paper II and III, we demonstrated that, in contrast to unstimulated EGFR and ErbB2, ErbB3 resides in intracellular vesicles in absence of added ligand. This is in line with previously reported granular cytoplasmic localization of ErbB3 in various human tissues (Lemoine et al. 1992; Prigent et al. 1992). We further showed in paper II that both endogenous, stably, and transiently expressed ErbB3, was internalized from plasma membrane in absence of added ligand and localized to early endosomes in several cell lines. In accordance with the constitutive internalization, also ErbB3 degradation did not depend on HRG. Degradation of ErbB3 was however slightly enhanced upon incubation with HRG-ECD. This is in consistence with previous reports showing that the constitutive turn-over rate of ErbB3 was increased by HRG-ECD, possibly due to ability of HRG-ECD to disrupt higher order oligomers of ErbB3 (Waterman et al. 1998; Warren et al. 2006). Based on data from paper II, we can also conclude that ErbB3 internalization does not depend on other members of ErbB family, nor does it depend on activation of c-Met. Whether ErbB3 is constitutively internalized as monomers, homodimers, or oligomers, however, still remains a question. By knocking down clathrin, we further demonstrated that ErbB3, similarly to EGFR, was endocytosed in a clathrin-dependent manner. However, while the ligand induced internalization of EGFR depends on kinase activity, ErbB3 was internalized in absence of added HRG, and interestingly, also upon inhibition of kinase activity. Thus, phosphorylation is not likely a signal for endocytosis of ErbB3. Nrdp1 is known to ubiquitinate ErbB3 in a constitutive manner, and it has been demonstrated that Nrdp1 is involved in quantity control through association with mainly the nascent form of ErbB3, although its role in ubiquitination and promoting degradation of the internalized mature ErbB3 is not excluded (Fry et al. 2011). It would therefore be of high interest to investigate the exact

mechanism of the clathrin-dependent endocytosis of ErbB3, with respect to involvement/requirement of Nrdp1, ubiquitination and potential adaptor proteins. What also still remains unclear is the fate of internalized receptor, whether and to what extent ErbB3 degradation depends on lysosomes, intramembrane cleavage, or proteasomes, and whether there is a pool of ErbB3 that enters the recycling route. ErbB3 has been postulated to be slowly internalized and recycled back to the cell surface (Waterman et al. 1998). The extent of ErbB3 recycling however remains unclear. Endocytosis and fast recycling has also been proposed for ErbB2, as a mechanism by which ErbB2 can maintain its predominant plasma membrane localization (Austin et al. 2004). However, based on what we show in papers II and III, the potential recycling of ErbB3, is not efficient enough to maintain a steady expression of ErbB3 at the plasma membrane. As demonstrated upon treatment with cycloheximide, a vast pool of ErbB3 was constitutively downregulated from the plasma membrane and degraded.

Reduced endocytosis of EGFR upon dimerization with ErbB3

We have previously shown that overexpression of ErbB2, which is inefficiently endocytosed, inhibits the EGF-induced downregulation of EGFR (Haslekas et al. 2005). In paper II we demonstrated that, even though ErbB3 is constitutively internalized, incubation with HRG did in fact slightly reduce the rate of EGF internalization. Since, HRG did not affect EGF internalization in PAE cells expressing only EGFR, the inhibitory effect required presence of ErbB3. Those data support EGFR-ErbB3 heterodimerization in PAE cells and suggest that EGFR-ErbB3 heterodimers are less efficiently downregulated from the plasma membrane than EGFR homodimers. How strong and persistent this inhibition is upon longer exposure to both ligands, as well as the mechanism of this inhibition, remains to be investigated. We additionally observed that expression of ErbB3 as such did not inhibit internalization of EGF. Nor did it inhibit the EGF-induced downregulation of EGFR upon 5 h treatment with cycloheximide. This is probably because ErbB3, as opposed to ErbB2, does not constitutively adapt an open conformation, and thus in absence of HRG, majority of ErbB3 does not constitutively dimerize with EGFR. Antiapoptotic and proliferative signaling from ErbB3-EGFR heterodimers was demonstrated to drive carcinogenesis (Liles et al. 2010). The HRG-mediated EGFR-ErbB3 heterodimerization, resulting in reduced downregulation of EGFR, could potentially contribute to prolonged EGF-induced signaling from the plasma membrane.

Inhibitory effect of ErbB2 on endocytic downregulation of ErbB3

While ErbB2 resides mostly at the plasma membrane, ErbB3 is internalized even in the absence of added ligand and without the need of kinase activity (paper II). We have previously shown that overexpression of ErbB2 inhibited EGF-induced internalization and degradation of EGFR, most probably due to EGFR-ErbB2 heterodimerization (Haslekas et al. 2005; Hughes et al. 2009). As demonstrated by Western blot in paper II, ErbB3 was degraded at a slower rate upon incubation with HRG in cells expressing high amounts of ErbB2, as compared to cells with little or no ErbB2. We therefore propose that upon ErbB2 overexpression, the HRG-stimulated ErbB3 dimerizes with ErbB2, which prevents its internalization and subsequent degradation. The inhibitory effect of ErbB2 on ErbB3 degradation was to some extent observed also in absence of ligand, arguing that some ErbB3 could form constitutive dimers with ErbB2. This is consistent with the observation that upon ErbB2 overexpression, ErbB2-ErbB3 dimers can form constitutively (Junttila et al. 2009). At the same time, in our confocal microscopy experiments, we could not observe that ErbB2 blocked the constitutive internalization of ErbB3 in PAE cells overexpressing ErbB2. This could suggest that, while some constitutive ErbB2-ErbB3 heterodimerization could take place in absence of ligand, the majority of ErbB3 was not engaged in heterodimers with ErbB2 and could be internalized. Alternatively it is possible that, while ErbB3 degradation was clearly reduced during 4 and 8 h period in cells overexpressing ErbB2, confocal microscopy was not sensitive enough to detect significant inhibition of ErbB3 internalization within 30 min period. While we did not demonstrate the direct association of ErbB2 with ErbB3, we observed that pertuzumab, which blocks ErbB2 dimerization, inhibited HRG-induced signaling and prevented the inhibitory effect of ErbB2 on ErbB3 degradation, similarly to what was earlier reported for EGFR (Hughes et al. 2009). Due to technical difficulties we could not directly show HRG-induced inhibition of ErbB3 internalization in cells overexpressing ErbB2, and there is a possibility that ErbB2-ErbB3 heterodimers get internalized and recycled back to the plasma membrane. Because in absence of GA, ErbB2 is not internalized, the recycling-scenario seems unlikely to explain reduced downregulation of ErbB3 in paper III.

ErbB2-ErbB3 heterodimer is highly oncogenic and reactivation of ErbB3 is responsible for acquired resistance to anti-EGFR and anti-ErbB2 therapy. Several mechanisms have been proposed to contribute to resistance to ErbB TKIs and among them are upregulation of ErbB3 at mRNA and protein level (Garrett et al. 2011; Grovdal et al. 2012) and relocation of ErbB3 to plasma membrane (Sergina et al. 2007; Takikita et al. 2011). Additionally, Beji et al. reported that ErbB3 was frequently found at the plasma membrane in colon cancer, which correlated with increased total level of ErbB3 and increased

phosphorylation of ErbB3 (Beji et al. 2011). Since ErbB2 and ErbB3 are often co-expressed in cancer, one could speculate that upregulation of the total and/or plasma membrane-localized ErbB3, could possibly be caused by heterodimerization with ErbB2. Our previous reports (Haslekas et al. 2005; Hughes et al. 2009) and data from paper III together suggest that ErbB2-mediated inhibition of endocytic downregulation could be a common mechanism by which ErbB2 affects downregulation of other ErbB family members.

Inhibition of HRG-signaling and induction of ErbB3 downregulation by pertuzumab in cells co-expressing ErbB2

Pertuzumab has been demonstrated to inhibit HRG-induced ErbB2-ErbB3 dimerization and signaling, but with varying efficiency and in a cell-type dependent manner (Lewis et al. 1993; Jackson et al. 2004; Cai et al. 2008; reviewed in Campbell et al. 2010). Our research group has previously demonstrated that pertuzumab, by preventing formation of EGFR-ErbB2 dimers, restored EGF-induced downregulation of EGFR in cells overexpressing ErbB2 (Hughes et al. 2009). In paper III we now show that pertuzumab prevented the inhibitory effect of ErbB2 on both the constitutive and the ligand-induced degradation of ErbB3. We propose that this was due to inhibition of ErbB2-ErbB3 heterodimerization, based on the demonstration that pertuzumab inhibited HRG-induced phosphorylation of ErbB3 in cells expressing ErbB2. While it efficiently reduced activation of ErbB3 in all cells expressing ErbB2, it affected downstream signaling with varying efficiency in various cell lines. The signaling inhibitory effect of pertuzumab, in contrast to that of trastuzumab, has been postulated to not depend on ErbB2 overexpression (reviewed in Friedlander et al. 2008). In fact, our results showed that HRG-induced downstream signaling was most efficiently inhibited in MCF-7 cells expressing low levels of ErbB2. In cells expressing high levels of ErbB2, pertuzumab, even at high concentrations, did not effectively block HRG-induced downstream signaling. This could be due to compensatory activation of EGFR-ErbB3 signaling, or a possible cross-talk of ErbB3 with c-Met or other RTKs. Furthermore, we observed that while HRG-induced signaling was efficiently blocked by pertuzumab in MCF-7 cells, the EGF-induced signaling remained unchanged. This implies that pertuzumab is likely to inhibit HRG-induced signaling from ErbB3-ErbB2, but in presence of EGF, EGFR homodimers seem efficient enough to sustain Erk and Akt activation. Pertuzumab had little effect on ErbB3 degradation, while it efficiently blocked HRG-induced signaling in MCF-7 cells. This suggests that due to the low expression level of ErbB2 in those cells, only a small amount of the total ErbB3 participates in ErbB2-ErbB3 heterodimerization. These heterodimers are efficient signal-activators, while the remaining majority of ErbB3 is

endocytosed and degraded as ErbB3 monomers or homodimers. This observation is also consistent with the notion that ErbB3 downregulation does not depend on its phosphorylation, as discussed in paper II.

ErbB3 kinase activity and signaling

Opposed to what initially was postulated, ErbB3 has recently been demonstrated to have a weak kinase activity (Shi et al. 2010; Telesco et al. 2011). In line with this, by using cells that did not express any other ErbB proteins, we were able to demonstrate that HRG to a small degree induced phosphorylation of ErbB3 and downstream effectors independently of other ErbB proteins (paper II). The phosphorylation of ErbB3 in those cells was not inhibited by neither Src, nor c-Met kinase inhibitors (data not shown), but it was blocked by AG1478, a kinase inhibitor that was known to target EGFR and ErbB2 (Yang et al. 2007). Those results, not only confirm that ErbB3 is kinase-active, but also argue that ErbB3 is able to form homodimers, in contrast to what has previously been postulated (Berger et al. 2004). In additional to HRG-induced phosphorylation of ErbB3, we observed a weak basal phosphorylation of ErbB3 that was also inhibited by the AG1478 kinase inhibitor (paper II). Based on this we conclude that ErbB3 has some kinase activity, and displays a low basal activation in absence of ligand. Consistent with this, a low constitutive activation of ErbB3 was present also in cells co-expressing EGFR, or ErbB2, or both (paper II and paper III). This ligand-independent basal activity of ErbB3 could possibly be explained by the finding that the C-terminal tail of ErbB3 lacks autoinhibitory function (Bublil et al. 2010). The constitutive phosphorylation of ErbB3 in cells overexpressing ErbB2 (paper III), is further consistent with reported ligand-independent formation of active ErbB2-ErbB3 heterodimers (Junttila et al. 2009). Efficient signal transduction of ErbB3 does, however, seem to depend on heterodimerization with other receptors (paper II and III and Shi et al. 2010) and on stimulation with HRG, which is thought to stabilize ErbB3-containing dimers (Junttila et al. 2009). Junttila et al. demonstrated that, while trastuzumab disrupted preexisting ErbB2-ErbB3 heterodimers, pertuzumab was most efficient in inhibiting HRG-induced ErbB2-ErbB3 heterodimers (Junttila et al. 2009). Based on this study, it was proposed that the interactions within ligand-dependent and ligand-independent heterodimers may be structurally different and mediated by distinct receptor-interfaces. We studied both EGFR-ErbB3 (paper II) and ErbB2-ErbB3 (paper III) signaling. Results from paper II indicate that EGFR-ErbB3 heterodimers efficiently induced activation of MAPK and PI3K pathways. However, while Erk phosphorylation was slightly stronger upon EGF stimulation, activation of Akt was most efficient in presence of HRG, which is in agreement with the direct binding of the p85 subunit of PI3K kinase to ErbB3. In paper III, pertuzumab efficiently blocked HRG-induced downstream signaling, but did not have detectable effect on the EGF-mediated signaling in MCF-7 cells.

Consequently, we conclude that in MCF-7 cells, in absence of EGF, the HRG-stimulated ErbB3, with its marginal kinase activity, depends on heterodimerization with the constitutively "open" ErbB2, to activate MAPK and PI3K pathways. However, in absence of HRG, the EGF-bound EGFR homodimers are efficient enough to sustain Erk and Akt activation in presence of pertuzumab. This again confirms the notion that while EGFR has an efficient kinase activity and forms strongly active homodimers, ErbB3 depends on heterodimerization for efficient activation of downstream signaling.

Concluding remarks

In summary, based on the results presented in this thesis, I propose that:

1. Cbl-mediated ubiquitination of EGFR promotes translocation of EGFR to CCPs and its subsequent internalization.
2. While kinase activity is essential for recruitment of Cbl to EGFR, ubiquitination *per se* can act as internalization signal, not excluding the possibility that under physiological conditions, several mechanisms may act in parallel to mediate EGFR endocytosis.
3. ErbB3, unlike EGFR and ErbB2, is internalized and degraded in absence of added ligand.
4. ErbB3, similarly to EGFR, is endocytosed in a clathrin-dependent manner, but unlike EGFR, it does not require kinase activity for internalization.
5. HRG and EGF-induced EGFR-ErbB3 heterodimerization reduces EGFR internalization.
6. Overexpression of ErbB2 inhibits degradation of ErbB3, and this inhibition can be prevented by incubation with pertuzumab.
7. Pertuzumab inhibits ligand-induced ErbB2-ErbB3 heterodimerization.
8. Pertuzumab reduces HRG-induced ErbB3 phosphorylation in cells co-expressing ErbB2 and reduces HRG-induced downstream signaling efficiently in cells expressing small amounts of ErbB2.
9. ErbB3 has some kinase-activity and can form homodimers, but depends on stimulation with HRG and heterodimerization for efficient signal transduction.

In spite of many similarities, ErbB proteins vary with respect to, among others, their intracellular localization, turnover rate, mechanism of downregulation, ligand-binding, kinase activity and signaling. Moreover, ErbB proteins influence each other. The behavior of one ErbB protein often depends on the relative level of expression of other members of ErbB family, and is often cell-type dependent. Consequently, drugs like for instance pertuzumab are likely to work in a cell- and tumor-dependent manner. In addition to the knowledge about general mechanisms controlling functions of ErbB proteins,

thorough molecular characterization of each tumor would potentially increase efficiency of the anti-ErbB cancer therapy. As The National Institute of General Medical Sciences (NIGMS, Bethesda, USA) states about basic research on their website: “It may take time to see significant advances; "miracle cures" are not the goal of this work. Sometimes, of course, the pieces come together and a real clinical breakthrough occurs. But the scientists' main purpose is to keep following the leads that appear most likely to yield missing pieces of information, even if the exact applications of the new knowledge are not immediately evident…From the body of knowledge and understanding amassed by basic researchers, clinical investigators can construct more rational and systematic ways to approach the problems presented by the diseases plaguing us today.”

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Paper I

Vibeke Bertelsen, Malgorzata Magdalena Sak, Kamilla Breen, Marianne S. Rødland, Lene E. Johannessen, Linton M. Traub, Espen Stang, and Inger Helene Madshus

A chimeric pre-ubiquitinated EGF Receptor is constitutively endocytosed in a clathrin-dependent, but kinase-independent manner.

Traffic, Vol. 12(4), 507-520, April 2011

Paper II

Malgorzata Magdalena Sak*, Kamilla Breen*,
Sissel Beate Rønning, Nina Marie Pedersen, Vibeke
Bertelsen, Espen Stang, and Inger Helene Madshus

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The oncoprotein ErbB3 is endocytosed in the absence of added ligand in a clathrin-dependent manner.

Carcinogenesis, Vol. 33(5), 1031-1039, May 2012

Paper III

Malgorzata Magdalena Sak, Monika Szymanska,
Vibeke Bertelsen, Max Hasmann, Espen Stang, and
Inger Helene Madshus

Pertuzumab counteracts the inhibitory effect of ErbB2 on degradation of ErbB3.

Submitted (Carcinogenesis)